UNIVERSITÉ DU QUÉBEC À MONTRÉAL

ÉTUDES DES CHANGEMENTS PHYSIOLOGIQUES ET MOLÉCULAIRES DU BLÉ (Triticum aestivum L.) EN RÉPONSE AUX CONCENTRATIONS ÉLEVÉES DE CO2 DURANT L'ACCLIMATATION AU FROID

THÈSE

PRÉSENTÉE

COMME EXIGENCE PARTIELLE

DU DOCTORAT EN BIOLOGIE

PAR

KHALIL KANE

DÉCEMBRE 2012

Je dédie ce travail :

A la mémoire de mon frère Seyni et de mon père.

A ma mère Codou Sow.

REMERCIEMENTS

A ma mère

A mes frères, James Bruce, Ndjido, Babacar Kane et Codou Sène

A mes cousines Codou et Mame Ngoné Sow

A mon directeur de thèse, le professeur Fathey Sarhan pour son encadrement et son support.

Au professeur Ragai Ibrahim, pour son dévouement à la science et son encadrement.

Au professeur Norman Hüner et Bernard Grodzinski pour leur soutien et expertise.

Au professeur Mario Houde pour sa contribution scientifique.

A mes chers collaborateurs Keshav Dahal et Amira Moheb.

Aux membres du jury de thèse, professeurs Normand Chevrier, Jean Danyluk et Patrick Gulick

Au professeur, François Ouellet

A mes amis, Mbaye Sall, Mbaye Diakhaté, Habiboulaye Sow, Angelo Dossou-yovo et Bénédicte Mateso

A mes collègues et amis de laboratoire, Amadou Diallo et Yero Seydi Ba

Aux Docteurs Mélanie Grondin, Zahra Agharbaoui, Mohamed Youssef Badawi ainsi qu'à tous mes collègues de laboratoire

A mes amis des groupes de recherche des professeurs Julie Lafond, Eric Rassart, Denis Archambault et Benoît Barbeau.

A Charles Lafaille, Aziz Salmone Fall, Dominique Zéphir et Rolland Amoussouga

A tous ceux qui m'ont permis de réaliser ce travail

TABLE DES MATIÈRES

LISTE DES FIGURES

LISTE DES TABLEAUX

LISTE DES ABRÉVIATIONS

CO_2	carbon dioxide
CA	cold acclimated
NA	non-acclimated
A_{sat}	light-saturated rates of CO_2 assimilation
R_{dark}	dark respiration rates
PPFD	Photosynthetic Photon Flux Density
J_{max}	maximum rate of photosynthetic electron transport
ETR	maximum rate of photosynthetic electron transport
COR	cold-regulated genes
EP	excitation pressure
Q	apparent maximum quantum efficiency for CO_2 assimilation
CE	carboxylation efficiency
C_i	internal leaf CO_2 concentrations

Gs	stomatal conductance
F_v/F_m	maximum PSII photochemical efficiency
WUE	water use efficiency
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
rbcL	gene encoding the large subunit of Rubisco
cFBPase	cytosolic fructose-1,6-bisphosphatase
Lhcb1	PSII light harvesting protein LHCB1
psaA	PSI reaction centre polypeptide, PsaA
psbA	PSII reaction centre polypeptide PsbA
Wcs120	wheat cold-specific protein, WCS120
Wcs19	wheat cold-specific protein, WCS19
Wcor410	wheat cold-regulated protein, WCOR410
WT	wild type

RÉSUMÉ

Les prédictions du réseau intergouvernemental sur les changements climatiques indiquent que la concentration ambiante de dioxyde de carbone (CO_2) d'environ 350 $\mu m.mol^{-1}$ va doubler d'ici la fin du siècle. Cette hausse de CO_2 combinée au réchauffement de la température va avoir un effet drastique sur l'agriculture et les écosystèmes. Grâce à leur grande variabilité génétique, le blé (*Triticum aestivum*) et le seigle (*Secale cereale*) sont très adaptés aux stress abiotiques et biotiques. Les variétés d'hiver capables de s'acclimater au froid sont plus résistantes aux basses températures. Cette meilleure capacité de tolérance pourrait-elle conférer un avantage durant la croissance et le développement des céréales soumis aux concentrations élevées de CO_2 de 700 $\mu m.mol^{-1}$? La comparaison des processus physiologiques, biochimiques et moléculaires associés à la réponse du blé et du seigle non acclimatés et acclimatés au froid durant leur croissance et développement en concentrations ambiantes et élevées de CO_2 ont été l'objet de notre étude. Dans le premier article de ce travail, nous avons comparé la contribution des effets de la plasticité phénotypique observée au niveau des feuilles et de la plante entière avec les effets biochimiques et moléculaires sur la performance photosynthétique et l'utilisation de l'eau des cultivars hivernaux et printanièrs de blé (cv Norstar et cv Katepwa) et de seigle (cv Musketer et cv SR4A) non-acclimatés (20 ^{0}C) et acclimatés au froid (5 ^{0}C). Le blé et le seigle d'hiver acclimatés au froid présentent des hausses de 22 et 44% de leur activité photosynthétique maximale (Asat) et du double de leur efficacité d'utilisation de l'eau. Les sucres produits par la photosynthèse sont exportés au niveau des organes de réserve et de la paroi cellulaire. Les analyses biochimiques et moléculaires montrent que les céréales d'hiver acclimatées au froid augmentent leur efficacité et taux de transfert d'électrons photosynthétiques, diminuent la pression d'excitation au niveau de leur photosystème II et augmentent la dissipation de l'énergie sous forme de chaleur, démontrant ainsi une meilleure performance face à l'inhibition photosynthétique associée au froid. Par ailleurs les résultats obtenus avec la surexpression de BNCBF17 chez *Brassica napus* indiquent que les gènes CBFs/DREBs semblent réguler la tolérance au gel et gouvernent l'architecture des plantes, l'anatomie des feuilles, la performance photosynthétique et l'efficacité de l'utilisation de l'eau. Nous discutons dans cet article des coûts et des bénéfices de la plasticité phénotypique en termes de survie hivernale et de la capacité de reproduction. Dans le deuxième article nous avons déterminé si l'exposition à court terme aux concentrations élevées de CO_2 de 700 $\mu m.mol^{-1}$ pouvait compenser l'inhibition photosynthétique induite par le froid chez

les variétés printanières de céréales. Les taux d'assimilation de la photosynthèse (Asat) ont été mesurés chez deux variétés (hivernale et printanière) de blé *Triticum aestivum* (cv Norstar et Katepwa) et de seigle *Secale cereale* (cv Musketeer et cv SR4A) non acclimatées (température 20/16 °C jour/nuit) et acclimatées au froid (Température 5/5 ^{0}C jour/nuit). Suite à une exposition courte de aux concentrations élevées de CO_2 de 700 µmol C mol^{-1}, les variétés printanières de blé et seigle acclimatées au froid présentent une diminution de 45 à 60% de leur taux de photosynthèse comparée à leurs homologues non acclimatées. Les plants de blé et de seigle d'hiver acclimatés au froid présentent une augmentation de 15 à 35% de leur photosynthèse comparativement aux plants non acclimatés. L'assimilation de CO_2 durant l'acclimatation au froid est 60% moins élevée chez les variétés printanières comparativement aux variétés hivernales. Ces résultats démontrent que l'exposition à court terme aux concentrations élevées de CO_2 ne peut compenser l'inhibition photosynthétique induite par le froid chez les variétés printanières. La limitation du CO_2 pour la Ribulose 1, 5-biphosphate carboxylase/oxygénase que l'on observe généralement à concentration ambiante de CO_2 est accentuée par l'acclimatation au froid chez les cultivars de printemps. De plus, l'exposition à court terme aux concentrations élevées de CO_2, ne permet pas aux cultivars de printemps d'ajuster la sensibilité thermale associée à la photosynthèse durant l'acclimatation au froid comparativement aux cultivars d'hiver. Dans le troisième article, l'analyse du transcriptome chez le blé (*Triticum aestivum*. L,) d'hiver Norstar cultivé à long terme en conditions de CO_2 ambiant (380 µmol C mol^{-1}) et élevé (700 µmol C mol^{-1}) a été initiée afin de déterminer les facteurs physiologiques et génétiques impliqués dans la réponse des plantes non-acclimatés (NA, 20°C) et acclimaté au froid (CA, 5 °C) aux concentrations élevées de CO_2. Les plantes acclimatées au froid en conditions de CO_2 ambiant présentent, un phénotype court et robuste, une réduction de 33% de leur croissance, une hausse double du poids spécifique de leur feuille de même que de leur quantité de protéine et une hausse de 30% de leur quantité de chlorophylle par unité de surface foliaire comparativement aux plantes non acclimaté. Les concentrations élevées de CO_2 ont peu d'effets sur ces paramètres morphologiques. Néanmoins les concentrations élevées de CO_2 ont entraîné une hausse de 30% de la biomasse des parties aériennes du blé non acclimaté et acclimaté au froid. Le blé Norstar acclimaté au froid maintient des taux de photosynthèses en conditions de lumière et de CO_2 saturantes comparables au blé non-acclimaté, mais requiert moins de quantum pour la fermeture du photosystème et pour la dissipation de l'énergie sous forme de chaleur. Les plantes non acclimaté et acclimaté au froid ne sont pas sensibles à l'inhibition photosynthétique induite par les concentrations élevées de CO_2. L'effet le plus marqué des concentrations élevées de CO_2 chez le blé non acclimaté est la diminution de l'expression des gènes impliqués dans la défense des plantes face aux pathogènes. Par contre, ces effets sont moins accentués chez les plantes acclimatées au froid grâce à l'induction des gènes impliqués dans la résistance aux pathogènes, dans la tolérance au gel, dans la protection et à la stabilisation du chloroplaste. Ces résultats

démontrent que l'acclimatation au froid et les concentrations élevées de CO_2 ont des effets opposés sur la régulation du système de défense des plantes. Ces résultats démontrent que es concentrations élevées de CO2 auront moins d'impact sur la performance et la productivité des plantes qui vivent dans les pays nordiques contrairement à celles qui vivent dans les environnements plus chauds. Avec les conditions de CO_2 élevé prévu, la sélection de plantes ayant des caractères de résistance aux pathogènes est fortement suggérée.

Mots clés : Assimilation de CO_2, concentrations élevées de CO_2, acclimatation au froid, transcriptome, stress abiotiques et biotiques, gènes de résistance aux pathogènes

CHAPITRE I

INTRODUCTION ET REVUE DE LITTERATURE

Il est bien établi que l'augmentation des gaz à effet de serre (GES) est responsable du réchauffement de la planète. Les émissions de GES sont mesurées en termes d'équivalent de CO_2 (éq. CO_2) qui représente la quantité d'un gaz particulier multiplié par son potentiel à réchauffer la planète. A peu près 1000 ans avant la révolution industrielle, la concentration de CO_2 atmosphérique était stabilisée à environ 270 $\mu mol.mol^{-1}$ (Prentice *et al.,* 2001). Aujourd'hui, la concentration de CO_2 est 38 % plus élevée (372 $\mu mol.mol^{-1}$). Selon les prédictions révélées par le réseau intergouvernemental sur le changement climatique (IPCC) cette valeur atteindra 550 $\mu mol.mol^{-1}$ au milieu de ce siècle et elle dépassera 700 $\mu mol.mol^{-1}$ à la fin du siècle. L'augmentation des teneurs en CO_2 ainsi que les changements climatiques auront des conséquences majeures pour l'agriculture et les écosystèmes naturels. Une bonne compréhension des effets des concentrations élevées de CO_2 ainsi que de leur combinaison avec d'autres facteurs associés au changement climatique est primordiale dans le but de contrer les effets néfastes sur les plantes.

Les effets de l'augmentation de la concentration de CO_2 sur la physiologie et le développement des plantes ont été bien documentés durant ces 20 dernières années (Stitt, 1991; Drake *et al.,* 1997; Norby *et al.,* 2002; Solomon *et al.,* 2007). A court terme les plantes terrestres simples de type C3 perçoivent et répondent directement à l'augmentation de la concentration de CO_2 par une augmentation du taux de carboxylation de l'enzyme Ribulose 1,5 biphosphate carboxylase/oxygénase (RUBISCO) et par une diminution de l'ouverture de leurs stomates. A long terme, une exposition prolongée des plantes aux concentrations élevées de CO_2 peut résulter en une acclimatation physiologique impliquant une diminution de leur capacité d'assimilation du CO_2 (Long *et al.,* 2004).

1.1 COMMENT LES PLANTES PERÇOIVENT ET RÉPONDENT À UNE ÉLÉVATION DE LA CONCENTRATION DE CO_2

Les plantes perçoivent les changements atmosphériques à travers leurs tissus exposés à l'air. A l'exception de quelques organes reproducteurs, seuls les organes photosynthétiques sont en contact direct avec l'atmosphère. Long *et al.,* (2004) ont montré que, seuls la surface interne des cellules de garde des stomates et le mésophylle peuvent percevoir directement un changement de la concentration de CO_2 atmosphérique.

Une courte exposition aux concentrations élevées de CO_2 peut entraîner : i) une diminution de la conductance et de la transpiration au niveau des stomates des feuilles ; ii) une amélioration de l'efficacité de l'utilisation de l'eau créant ainsi des

rendements photosynthétiques plus élevés et une meilleure efficacité d'utilisation de la lumière.

1.1.1 Conductance au niveau des stomates

Pour que la photosynthèse ait lieu, il faut que le CO_2 diffuse de l'atmosphère au site de carboxylation. La concentration de CO_2 au niveau du site de carboxylation (Cc) est plus faible que celle de l'atmosphère (Ca) à cause des différentes résistances que le CO_2 rencontre sous phase gazeuse ou liquide (Warren and Dreyer, 2006). Sous sa phase gazeuse, le CO_2 doit diffuser à travers la couche protectrice de la surface foliaire, à travers l'ouverture des stomates et aussi à travers l'espace entourant les cellules du mésophylle. Sous sa phase liquide, le CO_2 rencontre une résistance lorsqu'il entre dans les cellules du mésophylle et ensuite de la membrane chloroplastique au site de carboxylation (Aalto *et al.,* 1999). Les concentrations de CO_2 au niveau du site de carboxylation sont plus faibles que celles de l'intérieur (Ci). La conductance interne (gi) permet de décrire cette diminution de la concentration de CO_2 entre Ci et Cc en terme de photosynthèse nette (A) : $gi = A / (Ci - Cc)$. La conductance interne limite la photosynthèse (A) de 20% ou plus et a un grand effet sur les estimations *in vivo* du taux de carboxylation maximum (Vcmax) et des constantes de Michaelis-Menten (Km) pour la carboxylation et pour l'oxygénation (Epron, 1995; Curtis, 1998; Bernacchi *et al.,* 2007).

Les études rapportées par Curtis (1996) ainsi que par Drake et collaborateurs (1997) font état d'une diminution de 10 à 20% de la conductance en réponse à l'augmentation instantanée de la concentration de CO_2 de 372 à 700 $\mu mol.mol^{-1}$. Bien

que la plupart des angiospermes montrent une diminution progressive de la conductance de leurs stomates (gs) suite à une augmentation de la concentration de CO_2, il existe des exceptions. Les conifères sont insensibles aux variations de concentration de CO_2, de même que *Fagus sp* chez les angiospermes (Saxe, 1998).

Le dioxyde de carbone est une molécule non polaire et lipophile qui peut diffuser à travers la membrane plasmique. Des études sur la membrane chloroplastique ont révélé que les aquaporines (AQP) participent au transport transmembranaire du CO_2 pour la photosynthèse (Uehlein *et al.,* 2003; Flexas *et al.,* 2006). L'aquaporine NtAQP1 du tabac (*Nicotiana tabacum*) est une protéine qui a la capacité d'augmenter la perméabilité du CO_2 chez les oocytes de Xenopus (Nakhoul *et al.,* 1998). Cette propriété a fait de l'aquaporine une candidate idéale pour évaluer l'effet de la conductance du CO_2 au niveau du mésophylle en utilisant des plants déficients ou sur exprimant l'aquaporine NtAQP1. Les plantes déficientes en aquaporine montrent une diminution de 13% de leurs taux photosynthétiques tandis que les plantes surexprimant l'aquaporine présentent une augmentation de 20% comparée aux plantes contrôles sauvages. Ces recherches montrent également que la conductance du CO_2 au niveau du mésophylle est 30% plus basse chez les plants déficients et 20 % plus élevée chez les plantes qui surexpriment l'aquaporine comparée aux plantes contrôles de type sauvage. Ces résultats suggèrent une implication de l'aquaporine au niveau de la conductance des stomates.

1.1.2 Les sites de signalisation du CO2

La perception du CO_2 est une propriété des cellules de garde, qui sont sensibles aux concentrations de CO_2 intercellulaire plutôt que celles à la surface des cellules. Une augmentation du double de la concentration de CO_2 conduit à la

fermeture de 20 à 40 % de plus de stomates chez plusieurs espèces de plantes (Kim *et al.,* 2010). Le mouvement des stomates est régulé par la pression de turgescence au niveau des stomates. La concentration en ions et en solutés organiques contrôle la pression de turgescence. Les études physiologiques d'électrolytes ont montré que les concentrations élevées de CO_2 augmentent l'activité des canaux potassium (K^+) sortant, diminuent l'activité des canaux K^+ entrant, augmentent les activités des canaux anioniques de type S, stimulent la libération de chlorure (Cl^-) par les cellules de garde et augmentent la concentration de calcium au niveau des cellules de garde (McAinsh *et al.,* 1996; Brearley *et al.,* 1997; Hanstein and Felle, 2002; Raschke *et al.,* 2003). Ces changements causent une dépolarisation membranaire des cellules de garde et entraînent la fermeture des stomates.

1.1.3 Régulation des canaux ioniques

Les voies de signalisation précises qui fonctionnent en amont des canaux ioniques ne sont pas bien connues mais il a été suggéré que ces voies sont organisées sous forme de réseaux (Assmann, 1993; Schroeder *et al.,* 2001; Hetherington and Woodward, 2003). Il y a de multiples messagers potentiels durant la réponse des stomates au CO_2, incluant le calcium libre dans le cytosol, les gradients de pH, les canaux ioniques et le potentiel membranaire, les niveaux de zéaxanthine chloroplastique, l'ATP dérivant de la photosynthèse, la phosphorylation et la déphosphorylation des protéines apoplastiques et cytoplasmiques. Beaucoup de ces signaux s'entrecoupent avec la réponse des stomates face à l'acide abscissique et la lumière, suggérant que les cellules de garde utilisent de multiples mécanismes pour percevoir le CO_2 (Kim *et al.,* 2010). Néanmoins, il existe un dilemme à savoir si le site de perception du CO_2 se fait directement au niveau des cellules de garde (Roelfsema *et al.,* 2006; Lee *et al.,* 2008) ou par les cellules du mésophylle (Hedrich

and Marten, 1993; Mott *et al.,* 2008). L'idée que les deux types cellulaires (mésophylle et de garde) pourraient contribuer conjointement semble plus probable. L'identification des composants et des mécanismes spécifiques de signalisation du CO_2 par des approches génétiques apporterait plus de clarté sur la spécificité du type cellulaire et du mécanisme de signalisation du CO_2. Les recherches par criblage et caractérisation fonctionnelle dans les banques de mutants chez *Arabidopsis* ont permis l'identification des gènes et de leurs allèles qui interviennent dans le contrôle du mouvement des stomates (Kim *et al.,* 2010).

Le mutant *gca* (growth control by abscissic acid) a été identifié comme insensible à l'ABA chez *Arabidopsis* (Himmelbach, 1998). La fermeture des stomates induite lors des concentrations élevées de CO_2 (800 µmol.mol-1) chez les plantes normales n'est pas observée chez le mutant *gca*. De plus, les concentrations élevées de CO_2 n'ont pas non plus occasionné de changements de la concentration de calcium transitoire au niveau des cellules de garde (Young *et al.,* 2006) comparativement aux plantes sauvages. Ces résultats suggèrent que GCA2 fonctionne en aval du point de convergence du système de signalisation du CO_2 et de l'acide abscissique (Kim *et al.,* 2010).

Un autre mutant impliqué dans la perception du CO_2 au niveau des cellules de garde, implique la protéine kinase HT1. Elle a été suggérée comme régulateur des stomates en réponse aux concentrations élevées de CO_2 (Hashimoto *et al.,* 2006). Chez *Arabidopsis*, les deux mutants alléliques *ht1-1* et *ht1-2* sont altérés au niveau de leur habilité à contrôler le mouvement des stomates en réponse aux concentrations de CO_2. Le mutant dominant *ht1-2* présente une diminution marquée de sa réponse au CO_2 mais par contre, montre une réponse à la lumière bleue, à la fusicoccine et à l'acide abscissique (ABA) suggérant ainsi un rôle de HT1 dans le système de signalisation des mouvements des stomates en réponse au CO_2 qui serait indépendant

du transport de protons et de malate. Le gène *HT1* code pour une protéine kinase exprimée au niveau des cellules de garde. Les tests de phosphorylation démontrent que l'activité de la protéine kinase HT1 est respectivement fortement diminuée ou abolie chez les mutants *ht1-1* ou *ht1-2*. De plus, les plantes transgéniques exprimant l'allèle dominant négatif de *HT1* (K113W) n'ayant pas d'activité kinase, montrent une altération de leur réponse au CO_2. Ces découvertes indiquent que la protéine kinase HT1 est importante au niveau de la régulation du mouvement des stomates et sa fonction est plus liée à la réponse au CO_2 qu'à celle de l'ABA ou de la lumière.

Les effets de l'augmentation de la concentration de CO_2 sur la densité des stomates ont été étudiés chez *Arabidopsis* par Gray et collaborateurs (2000). Ces auteurs ont identifié le gène *HIC* (High carbon dioxide) qui code pour une protéine inhibitrice du développement des stomates en réponse aux concentrations élevées de CO_2. Le produit de ce gène *HIC* est l'enzyme 3-keto acyl coenzyme A synthase (KCS) impliquée dans la synthèse des chaînes longues d'acides gras. Les mutants *hic* présentent une augmentation de près de 42% de leur densité de stomates en réponse aux concentrations doublées de CO_2, comparativement aux plantes sauvages qui sont insensensibles au CO_2. La synthèse des chaînes longues d'acides gras des cires, des glycérolipides, des sphingolipides et des cutines végétales est assurée par les élongases. Chez les mutants *hic*, une déficience en KCS empêche la synthèse des composants de la matrice extracellulaire à la surface des cellules de garde. La perte des composants synthétisés par HIC se traduit en une augmentation de l'index et de la densité des stomates en réponse au CO_2 (Gray *et al.*, 2000). A l'opposé, les concentrations élevées de CO_2 n'ont pas affecté le développement des stomates chez les plantes contrôles sauvages. Ces résultats suggèrent que le gène *HIC* est impliqué dans le système de signalisation responsable du contrôle du nombre de stomates en réponse aux concentrations élevées de CO_2

Le gène *slac1* (slow anion channel-associated) code pour une protéine membranaire transporteur anionique qui est exprimée préférentiellement au niveau des cellules de garde. Elle est homologue à la protéine transporteur d'acide malique et de dicarboxylate des bactéries et champignons. La protéine SLAC1 est essentielle à la fermeture des stomates en réponse au CO_2, à l'acide abscissique, l'ozone, les transitions entre le jour et la nuit, le changement d'humidité, aux ions calcium, au peroxyde d'hydrogène et à l'oxyde nitrique. Les mutations chez SLAC1 affectent les courants au niveau des canaux anioniques de type S qui normalement sont activés par le calcium du cytosol et l'ABA. Par contre, les courants au niveau des canaux anioniques de type R ne sont pas affectés chez ces mutants. La faible homologie de SLAC1 avec les protéines transporteurs d'acide organique chez les bactéries et les champignons, ainsi que la perméabilité au malate des canaux anioniques de type S, suggèrent un rôle vital pour SLAC1 dans le fonctionnement des canaux anioniques de type S (Lee *et al.*, 2008).

La protéine membranaire ABC transporteur de malate au niveau des cellules de garde a été identifiée comme un inhibiteur de la fermeture des stomates induite par les niveaux de concentration élevée de CO_2. Chez *Arabidopsis*, les plantes déficientes en cette protéine (AtABC14) ferment beaucoup plus rapidement leurs stomates suite à l'exposition aux concentrations élevées de CO_2. Le malate qui se trouve au niveau de l'apoplaste est un des facteurs qui intervient dans la réponse des stomates face au CO_2. Par ailleurs, l'application exogène de malate a induit une réponse similaire à celle observée durant l'exposition aux concentrations élevées de CO_2. L'isolement de cellules de l'épiderme contenant uniquement des cellules de garde a montré que la fermeture des stomates par l'entremise du malate est beaucoup plus rapide au niveau des plantes transgéniques surexprimant AtABC14 comparées aux plantes sauvages. Ces résultats suggèrent que AtABC14 catalyse le transport du malate de l'apoplaste aux cellules de garde. De plus, lorsque AtABC14 est exprimée chez E.coli ou au

niveau des cellules HeLa, le transport de malate est augmenté. Il a donc été suggéré que la protéine AtABC14 module le mouvement des stomates par le transport du malate de l'apoplaste aux cellules de garde, ce qui augmente leur pression osmotique (Vahisalu *et al.,* 2008).

1.1.4 Augmentation de la photosynthèse

A la fin du 21 ième siècle, les concentrations de CO_2 au site actif de la RUBISCO chez les plantes de type C3 augmenteront de 6.3 à 15 µM selon les prédictions de l'IPCC. Cette hausse de la concentration de CO_2, entraînera une augmentation du taux et de l'efficacité de la photosynthèse pour deux raisons.

Premièrement vu qu'aux concentrations de CO_2 actuelles d'environ 350 ppm, l'enzyme RUBISCO est présente en beaucoup plus grande quantité comparée à son substrat le CO_2, l'augmentation de la concentration de CO_2 augmenterait le taux de carboxylation.

Deuxièmement, une augmentation de la concentration de CO_2 aura comme effet de mettre en concurrence les réactions de carboxylation et d'oxygénation de la RUBISCO, ce qui diminuerait la perte de CO_2 et le coût énergétique associé au flux de 2-phosphoglycolate à travers la photorespiration (Gray *et al.,* 2000). Connaissant la spécificité moyenne de la RUBISCO, ses Km pour le CO_2 et l'O_2, de même que les constantes interne (Ci) et externe (Ca) de concentration de CO_2, il est possible de calculer l'absorption de CO_2 qui résulterait de l'augmentation de la concentration de CO_2 atmosphérique (Leavitt *et al.,* 1995; Bryant *et al.,* 1998). A une température de 25°C, une augmentation de la concentration de CO_2 atmosphérique de 372 $\mu mol.mol^{-1}$ à 550 $\mu mol.mol^{-1}$ devrait entraîner une augmentation de l'activité de la RUBISCO et

de la teneur en ribulose-1,5-biphosphate au niveau des feuilles de 36 et 12% respectivement. Pour une concentration de CO_2 de 700 $\mu mol.mol^{-1}$, cette augmentation serait de 63 et 18 % respectivement (Bernacchi *et al.,* 2003).

En résumé, les plantes C3 simples perçoivent et répondent directement à l'augmentation de la concentration de CO_2 à travers les effets directs de l'augmentation de la carboxylation par la RUBISCO et la diminution de l'ouverture des stomates. Pour une plante qui croît en isolement, l'augmentation de la concentration de CO_2 par l'augmentation de l'efficacité de l'utilisation de la lumière dans l'absorption nette de CO_2, résulte en une augmentation de la croissance et donc une augmentation de la production de feuilles. Ceci est renforcé par une diminution de l'utilisation de l'eau qui, plus tard, accélère le développement des feuilles. Chez le blé, *Triticum aestivum*, le gain net de carbone par la photosynthèse est estimé à plus de 13% durant l'exposition aux concentrations élevées de CO_2 comparées aux concentrations ambiantes. Chez le riz, *Oryza sativa*, il est de 9% (Ainsworth and Long, 2005).

L'augmentation de la concentration de CO_2 n'aura pas d'effet direct sur les plantes de types C4 vu que ces dernières évitent la photorespiration et sont déjà saturées en CO_2 aux concentrations de CO_2 actuelles grâce à leur mécanisme de concentration (Sage *et al.,* 1989).

1.1.5 Contrôle moléculaire de l'activité de la RUBISCO

Afin de bien fonctionner, la RUBISCO doit être activée par une réaction de carbamylation réversible au niveau de son résidu lysine et par la liaison d'ions magnésium (Mg^{2+}) (Cleland *et al.,* 1998). La carbamylation est catalysée par la RUBISCO activase qui permet la dissociation des sucres phosphatés. L'activité de la protéine chaperone RUBISCO activase requiert de l'ATP. La régulation de la

RUBISCO activase n'est pas entièrement comprise, mais il semblerait que le ratio ATP:ADP ainsi que le potentiel rédox seraient des mécanismes de régulation chez quelques espèces (Portis, 2003). Aux concentrations actuelles de CO_2 et de luminosité, la RUBISCO est carbamylée et complètement active (Von Caemmerer and Quick, 2000). Avec l'augmentation de la teneur en CO_2, la fixation de carbone augmente aussi, ce qui entraîne une hausse de la demande en ATP nécessaire à la régénération du ribulose-1,5-bisphosphate (RubP). La photosynthèse devient donc beaucoup plus limitée par la régénération du RubP que par la RUBISCO. Les réductions du ratio ATP:ADP au niveau du chloroplaste entraînent donc une baisse de l'activité de la RUBISCO activase. Les réductions de l'activité de la RUBISCO activase ont été observées chez plusieurs espèces de plantes exposées à des concentrations élevées de CO_2. Par contre, les effets de la réduction de l'activité de la RUBISCO ne sont observés que durant les périodes courtes d'exposition au CO_2 (Rowland Bamford *et al.,* 1991; Stitt, 1991; Drake *et al.,* 1997; Moore *et al.,* 1999). La régulation du contenu en RUBISCO se fait par des mécanismes qui touchent la transcription, la post-transcription, la traduction et la post-traduction (Stitt and Krapp, 1999; Smeekens, 2000; Rolland *et al.,* 2002; Long *et al.,* 2004).

1.1.6 Effets directs des concentrations élevées de CO2 sur la respiration diurne

Les premières études des effets de la concentration de CO_2 élevée sur les plantes font état d'une diminution de la respiration suite à une élévation de la concentration de dioxyde de carbone. Dans ces études, la respiration est diminuée de près de 20% suite à une augmentation double de la concentration de CO_2. Ces effets ont été observés dans plusieurs types de tissus incluant les feuilles, les racines, et les tiges (Drake *et al.,*1997). A partir des années 2000, cette diminution de la respiration a été associée à un artéfact de mesure par un nouveau courant de pensée basé sur des approches techniques apparemment plus fiables dans la mesure de l'oxygène dégagée (Amthor, 2000; Jahnke, 2001; Jahnke and Krewitt, 2002). Ce nouveau courant de

pensée est toujours en opposition avec les études qui continuent de montrer une diminution de la respiration malgré les ajustements apportés au niveau de la mesure du dégagement d'oxygène. Chez le riz, une diminution de 19% de la respiration a été observée au niveau des feuilles suite à une augmentation de la concentration de CO_2 de 350 à 700 µmol mol^{-1} (Baker *et al.,* 2000). Chez le pin (*Pinus sylvestris*), il y a une diminution de la respiration de 31 et 79% (Jach and Ceulemans, 2000). D'autres études réalisées sur huit espèces de plantes sauvages et herbacées sauvages, montrent une diminution de la respiration de 16 à 40 % suite à une exposition au CO_2 de plus de 60 à 1000 µmol.mol^{-1} (Bunce, 2001). Par ailleurs, les études sur 12 espèces herbacées différentes ont rapporté une diminution de 11% de la respiration au niveau de feuilles suite à une exposition de 360 à 1300 µmol.mol^{-1} (Tjoelker *et al.,* 2001). Chez le soya, cette diminution est de 18% lorsque la concentration de CO_2 est augmentée de 350 à 1400 ppm (Bunce, 2001).

Un mécanisme plausible, qui pourrait être responsable de cet effet direct, est l'inhibition des enzymes du système de transport d'électrons des mitochondries. Des expériences *in vitro* ont montré que les concentrations élevées de CO_2 diminuent les activités de sa cytochrome déshydrogénase et de la succinate déshydrogénase (Reuveni and Gale, 1985; Drake *et al.,* 1997). Les études sur des cals de feuilles d'oeillet (*Dianthys caryophyllus*) ont montré que la cytochrome C oxidase est inhibée par la forte concentration de dioxyde de carbone (Gonzales *et al.,* 1996). La possibilité que cette inhibition des enzymes par le CO_2 est directement liée à la concentration de dioxyde de carbone sur la respiration des plantes est appuyée par les mesures sur différents types d'organelles et de tissus de plantes (Palet *et al.,* 1991). Des études *in vivo* au niveau des tiges excisées de *S. olneyi* et chez le soya ont montré que le fait de doubler la concentration de CO_2 diminue l'absorption d'O_2 (Drake *et al.,* 1997).

1.2 EFFETS CHEZ LES PLANTES D'UNE LONGUE EXPOSITION À DES CONCENTRATIONS ÉLEVÉES DE CO2

Une caractéristique commune des plantes exposées aux concentrations élevées de CO_2 au niveau des chambres de croissance est l'incapacité de celles-ci de soutenir la stimulation initiale et maximale de l'absorption nette de CO_2 sous les conditions optimales de microclimat (Drake *et al.,* 1997). L'exposition à long terme des plantes aux concentrations de CO_2 élevées peut résulter en une acclimatation physiologique impliquant une diminution dans leur capacité d'assimilation du CO_2 (Peet *et al.,* 1986). La réduction de la capacité de carboxylation découlant de la croissance des plantes en présence de concentrations élevées de CO_2 est souvent associée à une diminution de la quantité de RUBISCO et des niveaux de transcrits de sa petite sous-unité rbcS, ainsi que d'autres gènes photosynthétiques (Moore *et al.,* 1999). Cette acclimatation est fréquemment associée à l'augmentation de l'amidon et de la concentration d'hydrates de carbone solubles au niveau des feuilles sources (Delucia *et al.,* 1985).

1.2.1 Mécanismes par lesquels, les concentrations élevées de CO2 régulent la capacité photosynthétique

La régulation de la photosynthèse durant l'exposition aux concentrations élevées de CO_2 des plantes C3 est apparemment très variable et les raisons de cette variation au niveau de la réponse aux concentrations élevées de CO_2 sont incertaines et très débattues (Moore *et al.,* 1999; Ainsworth *et al.,* 2002).

1.2.2 Accumulation des hydrates de carbone

L'augmentation des teneurs en hydrates de carbone est le changement universel le plus prononcé et observé chez les plantes C3 qui croissent en présence de concentration élevée de CO_2 (Drake *et al.,* 1997). Le saccharose est le produit majeur des cellules photosynthétiques, c'est sous cette forme que le carbone est transporté

dans la plante. Donc, le contenu en saccharose dans les feuilles va refléter la balance de l'apport photosynthétique et des demandes au niveau de la croissance, de l'emmagasinage ainsi que de l'assimilation des nutriments (Farrar *et al.,* 2000). Le sucre n'est pas seulement un substrat, mais a aussi un rôle régulateur au niveau du contrôle de l'expression de plusieurs gènes chez les plantes. Donc l'hypothèse que l'excès d'hydrates de carbone observé durant l'exposition aux concentrations élevées de CO_2 puisse rétroagir sur l'expression de gènes photosynthétiques et mener ainsi à l'acclimatation est attrayante (Koch, 1996).

La répression de gènes photosynthétiques par l'augmentation de la concentration d'hydrates de carbone solubles pourrait expliquer l'acclimatation photosynthétique durant l'exposition aux concentrations de CO_2 élevées.

Le saccharose est la forme d'hydrate de carbone qui est exportée des feuilles chez la plupart des plantes terrestres, et il est aussi une des formes sous laquelle les sucres sont stockés dans les vacuoles. Lorsque la photosynthèse excède la capacité d'utilisation et d'exportation des hydrates de carbone, le saccharose s'accumule dans le phloème et les vacuoles du mésophylle des feuilles. Le modèle proposé par Moore et collaborateurs (1999) est très convaincant sur le contrôle moléculaire de la quantité de RUBISCO via le cycle du saccharose durant l'exposition aux concentrations élevées de CO_2. Selon ces auteurs, l'excès de saccharose de la photosynthèse est hydrolysé par des invertases vacuolaires et apoplastiques, et les hexoses en dérivant sont phosphorylés par des hexokinases. Les produits sont ensuite réutilisés pour la synthèse de saccharose. Le glucose qui provient de la conversion du maltose en saccharose par l'amylomaltase, sert de substrat aux hexokinases. Une hexokinase pourrait être liée à l'enveloppe externe de la membrane chloroplastique procurant de ce fait un signal permanent de système de perception d'hexokinase (Wiese *et al.,* 1999). Quelques études ont partiellement détaillé le système du signal de transduction

qui lie l'hexokinase à la diminution de la capacité photosynthétique (Smeekens, 2000; Rolland *et al.,* 2002).

La perte de la capacité photosynthétique à travers l'acclimatation, particulièrement la quantité et l'activité de la RUBISCO, est très probablement expliquée par la diminution de l'expression de gènes photosynthétiques spécifiques ou de produits de gènes en réponse à l'augmentation de la production de saccharose à l'intérieur des cellules du mésophylle. C'est le phénomène observé, lorsque la photosynthèse excède la capacité d'exportation et d'utilisation des hydrates de carbone.

Les mécanismes de contrôle initiés par les hydrates de carbone varient au niveau des espèces, mais ils ciblent tous la petite sous-unité de la RUBISCO à travers un contrôle de la transcription ou de la traduction en interférant avec l'assemblage de l'holoenzyme (Moore *et al.,* 1999). Cette réponse est aggravée par les limitations génétiques, comme le déterminent des modèles de croissance, et des limitations environnementales, telles que l'insuffisance en azote ou les basses températures (Leakey *et al.,* 2009).

Néanmoins, le contrôle moléculaire par les hydrates de carbone n'est pas observé chez le blé de printemps exposé à des concentrations élevées de CO_2 de 550 $\mu mol.mol^{-1}$ (Nie *et al.,* 1995). La relation entre les concentrations d'hydrates de carbone (glucose-6-P, glucose, fructose, saccharose, fructanes, amidon) et les niveaux de transcrits d'ARNm des gènes de l'apoprotéine du photosystème I, de la sedoheptulose-1,7-biphosphatase, de la phosphoribulokinase, de la phospho-glycérokinase, et des sous-unités large et petite de la RUBISCO au niveau des feuilles a été examinée à des stades de développement différents ainsi que durant le cycle diurne. En tout, une faible corrélation entre l'augmentation de la concentration

d'hydrates de carbone solubles et la diminution des niveaux de transcrits des gènes nucléaires a été observée. La concentration en hydrates de carbone solubles entre les feuilles exposées à des concentrations élevées et ambiantes de CO_2 a diminué au cours du développement à cause du remplissage des graines, tandis que les niveaux de transcrits ont augmenté (Nie *et al.,* 1995).

Il y a des évidences expérimentales d'un lien entre le signal par le sucre avec plusieurs autres composés probables, en particulier, le nitrate et l'ABA (Stitt and Krapp, 1999; Kaiser and Huber, 2001; Rook *et al.,* 2001).

1.2.3 Contenu en azote

Une autre possibilité est que l'acclimatation soit le résultat d'une diminution non sélective du contenu en azote. Les études de Makino et collaborateurs (1997) sur des plants de riz ont montré que la réponse de la croissance des plantes face aux concentrations élevées de CO_2, est contrôlée par l'expansion de la feuille ainsi que par l'attribution d'azote au niveau de la plante. Dans ce modèle, la diminution observée de la RUBISCO reflète une diminution générale des protéines de la feuille à cause de la relocalisation de l'azote dans les plantes ou plutôt durant la sénescence des feuilles de plantes carencées en azote (Makino *et al.,* 1997; Nakano *et al.,* 1997). Sous les conditions de limitation d'azote, l'acclimatation en concentrations élevées de CO_2 pourrait s'accélérer, car les plantes sont plus grandes et donc devraient expérimenter la limitation aigue en azote plus tôt, ou à une plus grande échelle. Farage et collaborateurs (1998) ont démontré que même si la croissance est affectée par la faible concentration d'azote, l'acclimatation photosynthétique durant l'exposition aux concentrations élevées de CO_2 peut être améliorée si l'azote est ajouté directement durant la croissance des plantes, ce qui supporte le concept qui stipule que c'est la dilution de l'azote qui entraînerait l'acclimatation de la RUBISCO et non la demande en azote. Des études plus récentes par Bloom et collaborateurs

(2010) chez *Arabidopsis* et chez le blé ont montré que l'exposition aux concentrations élevées de CO_2 inhibe l'assimilation du nitrate qui est la forme prédominante d'azote disponible pour les plantes. Par conséquent, l'inhibition de l'assimilation du nitrate par les concentrations élevées de CO_2 devrait mener à une diminution de la production des composés organiques azotés. Ceci pourrait être responsable de la diminution de 11 et de 20% respectivement des protéines de blé et d'*Arabidopsis* observée dans les études par l'enrichissement en CO_2 à ciel ouvert (FACE) où les plantes ont été exposées à des concentrations élevées de CO_2 sous une longue période de temps (Kimball *et al.,* 2001; Kimball *et al.,* 2002; Hôgy *et al.,* 2009). Puisque l'influence de l'enrichissement en CO_2 sur le contenu en azote au niveau des feuilles est fortement corrélée avec son influence sur la photosynthèse et la croissance, il est à supposer que l'inhibition de l'assimilation du nitrate par le CO_2 et la diminution résultante du contenu en azote pourraient jouer un rôle majeur dans le phénomène d'acclimatation au CO_2 avec la diminution de la photosynthèse et de la croissance des plantes C3 après une longue exposition aux concentrations élevées de CO_2.

D'après les études de Bloom et collaborateurs (2010), il y a au moins trois mécanismes physiologiques qui pourraient expliquer la relation entre les concentrations élevées de CO_2 et l'assimilation du nitrate.

Un des mécanismes implique la première étape biochimique de l'assimilation du nitrate qui est la conversion de NO_3^- en NO_2^- au niveau du cytoplasme des cellules du mésophylle des feuilles. La photorespiration stimule l'exportation de l'acide malique des chloroplastes et augmente la disponibilité de la forme réduite de NADH cytosolique qui alimente la conversion de NO_3 en NO_2^- (Bloom *et al.,* 2010). Les concentrations élevées de CO_2 ou la faible concentration d'O_2 atmosphérique diminuent la photorespiration, ce qui entraîne une diminution de la disponibilité du

pouvoir réducteur nécessaire pour la réduction du nitrate. Au contraire, le cycle de fixation de carbone des plantes de type C4 génère suffisamment d'acide malique et de NADH au niveau du cytosol des cellules du mésophylle. Ceci explique pourquoi l'assimilation du nitrate au niveau des feuilles est relativement indépendante de la concentration de CO_2 chez les plantes de type C4 et limité au niveau du mésophylle (Rathnam and Edwards, 1976; Cousins and Bloom, 2003).

Un autre mécanisme physiologique qui pourrait relier l'assimilation du nitrate et les concentrations élevées de CO_2 est la translocation du NO_2^- du cytosol au chloroplaste. Il y a au moins six transporteurs de la famille des NarI impliqués dans la translocation du NO_2^- du cytosol au chloroplaste chez *Chlamydomonas* et quelques-uns transportent le NO_2^- et le HCO_3^- (Mariscal *et al.,* 2006). D'autres études ont démontré que le HCO_3^- inhibe l'influx de NO_2^- dans les chloroplastes de blé et du pois, ce qui indique l'existence d'un autre système analogue qui fonctionnerait au niveau des plantes supérieures (Bloom *et al.,* 2002). Durant l'exposition aux concentrations élevées de CO_2, un influx de NO_2^- plus lent au niveau des chloroplastes diminuerait l'assimilation de nitrate.

Le troisième mécanisme physiologique liant l'assimilation du nitrate et les fortes concentrations de CO_2, implique la compétition pour les pouvoirs réducteurs au niveau du stroma des chloroplastes. La fixation de carbone des plantes C3, la réduction de NO_2^- en NH_4^+ ainsi que l'incorporation d'ammonium en acides aminés requiert de la ferrodoxine réduite qui est générée par la chaîne de transport photosynthétique. Les enzymes impliquées dans ces processus ont des affinités différentes pour la ferrodoxine réduite : la Ferrodoxine-NADP réductase (FNR) a une constante d'affinité (Km) de 0.1 µM, la nitrite réductase a un Km de 0.6 µM et la glutamine synthase (GOGAT) a un Km de 60 µM (Ort and Yocum, 1996). Par conséquent, l'assimilation du nitrate ne peut se poursuivre que si la disponibilité de la

ferrodoxine réduite excède celle nécessaire pour la formation de la forme réduite de NADPH (Bloom *et al.*, 2010).

1.2.4 Effets des concentrations élevées de CO2 sur la floraison

La transition du stade de croissance végétatif à celui de la floraison est une étape critique dans le cycle de vie des plantes. Le moment de la floraison est coordonné par des facteurs environnementaux et endogènes incluant l'eau, la disponibilité des nutriments, la photopériode et la température (Bernier and Périlleux, 2005; Bäurle and Dean, 2006). Le moment de la floraison est donc crucial pour la reproduction des plantes afin d'assurer leur pérennité. L'augmentation de la concentration de CO_2 jouerait un rôle important dans la détermination de cette réponse. Par exemple, les études de Cleland *et al.*, (2006) sur des espèces herbacées terrestres ont montré que l'augmentation de température d'environ 1.5°C permet d'accélérer la floraison de 2 à 5 jours, tandis que ces mêmes plantes retardent leur floraison de 2 à 7 jours lorsqu'elles sont exposées à des concentrations de CO_2 de 680 ppm. Lorsque ces deux traitements sont combinés, les concentrations de CO_2 annulent les effets de la floraison précoce observée aux températures élevées. Les effets de la concentration de CO_2 sur la floraison rapportés par 60 études qui se sont intéressées à 90 espèces de plantes différentes sont très variables. Plus de 57% des espèces sauvages et 62% des espèces cultivées analysées montrent un changement significatif du moment de leur floraison incluant un retard et/ou une accélération lorsqu'elles sont exposées à des concentrations de CO_2 de 600-1000 ppm. Par contre, certaines espèces telles que le blé de printemps (*Triticum aestivum* cv Sport) et l'avoine (*Avena sativa* cv Kapp) ne montrent aucun changement au niveau de leur temps de floraison lors de l'exposition à des concentrations élevées de CO_2 (Springer and Ward, 2007).

Peu d'informations sur les mécanismes primaires des effets de la concentration élevée de CO_2 sur la floraison sont disponibles. Springer et Ward (2008) ont analysé chez deux écotypes d'*Arabidopsis* les effets des concentrations élevées de CO_2 sur la floraison. L'écotype SG, issu de croissements entre écotypes performants en conditions de CO_2 élevées, présente un retard de floraison significatif lorsqu'elle est exposée à des concentrations de CO_2 élevées. Par contre le second écotype, issu aussi du même croisement que SG, n'est pas affecté au niveau de sa floraison par les concentrations élevées de CO_2. Des analyses moléculaires ont montré que les concentrations élevées de CO_2 influencent l'expression des gènes de floraison. De plus, le retard de floraison observé chez la variété adaptée au CO_2 est associé à l'expression soutenue du gène répresseur de la floraison appelé *FLC* (Flowering Locus C). Le gène *FLC* est un répresseur de la floraison et la diminution de sa transcription entraîne une augmentation des gènes *SOC1* et *LEAFY* (*LFY*) qui sont des activateurs de la floraison (Springer *et al.,* 2008).

Chez Arabidopsis, le contrôle de la transition pour la floraison fait appel à plusieurs voies de signalisation. Ces voies de signalisation ont toutes comme point commun les gènes du méristème *LFY* et *APETALA* 1 (*AP1*) (Bernier and Périlleux, 2005; Bäurle and Dean, 2006).

La première voie de signalisation favorisant la floraison est celle de la lumière. Les changements de photopériode ainsi que la qualité de la lumière sont des facteurs qui influencent le temps de la floraison. Les deux autres voies de signalisation impliquant la température, mais agissant de façon indépendante, sont la vernalisation et la thermosensibilité. La capacité à vernaliser chez les plantes est un caractère epigénétique et épistatique. Les variétés qui vernalisent requièrent de longues périodes d'exposition aux basses températures avant de pouvoir fleurir, ce qui prévient la floraison durant l'hiver (Danyluk *et al.,* 2003). La voie de

thermosensibilité altère le moment de la floraison en réponse aux changements de la température ambiante de croissance (Blázquez *et al.,* 2003). Une autre voie de signalisation de la floraison est la voie autonome, elle contrôle la floraison en réprimant le gène *FLC* comme le font la vernalisation et la thermosensibilité (Michaels and Amasino, 1999; Blázquez *et al.,* 2003; Simpson, 2004).

Contrairement aux concentrations élevées de CO_2, l'effet de la température sur la transition florale est un facteur qui a retenu l'attention durant ces dernières années et plusieurs des gènes impliqués ont été très bien caractérisés chez le blé et *Arabidopsis* (Blázquez *et al.,* 2003; Danyluk *et al.,* 2003; Kane *et al.,* 2005; Bäurle and Dean, 2006; Kane *et al.,* 2007).

1.2.5 Effets des concentrations élevées de CO2 sur le rendement en grain du blé

Il existe de nombreuses études portant sur les effets des concentrations élevées de CO_2 sur le rendement en grains du blé (Amthor, 2000). Ces études ont été divisées en 5 catégories selon les méthodes de contrôle du CO_2: 1) chambre de laboratoire, 2) serre, 3) chambre de croissance à ciel ouvert ou 4) fermée et 5) enrichissement en CO_2 dans les champs. Pour une meilleure analyse des effets des concentrations élevées de CO_2 sur le rendement, seules les études dont la concentration en CO_2 est contrôlée durant la majorité ou la totalité du cycle de vie du blé ont été retenues (Amthor, 2000). Lorsque l'apport en nutriments et en eau étaient suffisants et que la température était favorable, les concentrations élevées de CO_2 allant jusqu'à 2000 ppm ont entrainé une augmentation du rendement avec un effet maximal de 37% à une concentration de 890 ppm. En moyenne, le fait de doubler la concentration de CO_2 de 350 à 700 ppm induit une augmentation de 31% du rendement. Néanmoins des variations au niveau du rendement ont été observées entre les 5 méthodes à cause des interactions entre les concentrations élevées de CO_2 avec d'autres facteurs tels que l'apport en nutriments et en eau, la concentration en ozone et la température. La

hausse du rendement en présence de concentrations élevées de CO_2 était moins élevée lorsque l'apport en nutriments était limitant. Lorsque la limitation était plus sévère, les concentrations de CO_2 ont entrainées une diminution du rendement. Aussi, des concentrations de CO_2 de plus de 2000 ppm ont entrainé une diminution du rendement même avec un apport suffisant en nutriments. Les concentrations élevées de CO_2 ont stimulé le rendement des plantes exposées à un stress hydrique modéré, mais n'ont pas compensé les effets d'une sécheresse (Pinter *et al.,* 2000). Des études plus récentes ont montré qu'une augmentation de la concentration de CO_2 de 358 à 712 ppm entraîne une augmentation de 6,1 % du rendement en grains de blé chez les plants suffisamment arrosés. Lorsque les plants étaient exposés à une sécheresse, le rendement en grain augmentait de 10 % (Qiao *et al.,* 2010).

Les concentrations élevées d'ozone ont réduit les effets positifs des concentrations élevées de CO_2 sur le rendement du blé. Une diminution de 6 à 10% du rendement et de 3 à 6 % du poids des grains a été observée avec l'application additionnel d'ozone chez des plants exposés aux concentrations élevées de CO_2 (Pleijel *et al.,* 2000). Un réchauffement de 1 à 4 ^{0}C a contrecarré les effets positifs des concentrations élevées de CO_2 sur le rendement (Amthor, 2000).

Tous ces résultats suggèrent que la combinaison des concentrations élevées de CO_2 avec d'autres facteurs entraîne des réponses variées.

1.3 EFFETS DES CONCENTRATIONS ÉLEVÉES DE CO_2 SUR L'EXPRESSION DES GÈNES

Bien qu'il y ait beaucoup d'informations sur les effets physiologiques associés à l'augmentation des concentrations élevées de CO_2, il y a peu d'études sur l'expression à grande échelle des gènes. La technique des micropuces d'ADN a été utilisée chez quelques espèces de plantes telles que le soya, *Arabidopsis*, le riz, le

peuplier et le fraisier, afin de déterminer leur profil d'expression durant l'exposition aux concentrations élevées de CO_2.

1.3.1 Chez Arabidopsis

Les effets des concentrations élevées de CO_2 sur le transcriptome de deux écotypes d'*Arabidopsis*, cv Columbia et cv Cape verde sélectionnés sur la base de leur performance face à divers stress environnementaux, ont été analysés par la technique des micropuces (Li *et al.*, 2008). Les analyses ont révélé que 213 transcrits étaient régulés à la baisse et 296 régulés à la hausse chez les deux écotypes exposés à des concentrations élevées de CO_2 (550 ppm). Les transcrits régulés à la baisse sont reliés aux processus de la photosynthèse, incluant les réactions photochimiques, le cycle de Calvin et la photorespiration avec notamment les transcrits des sous-unités des photosystèmes I et II (PsaK, PsaN, PsbQ, PsbW, PsbX et PsbO2) les transcrits des antennes collectrices de lumière et du transport des électrons. La glycéraldehyde-3-phosphate déshydrogénase, dont les produits sont connus pour interagir avec les protéines CP12 *in vivo* au niveau du cycle de Calvin, le complexe de la NADH déshydrogénase impliqué dans la respiration et le transport des électrons au niveau du photosystème I et la glycine décarboxylase ont été régulés à la baisse. Le développement des chloroplastes et la composition en lipides ont aussi été affectés avec les diminutions significatives des transcrits de la protéine transporteur d'acyl impliquée dans la biosynthèse des acides gras de la membrane chloroplastique et le transcrit de la protéine désaturase d'acides gras lié à la composition des lipides du thylacoïde. De façon générale, les gènes ayant des fonctions au niveau du chloroplaste ont été régulés à la baisse.

Les gènes régulés à la hausse sont principalement impliqués dans le métabolisme et l'utilisation du carbone. Les plus fortement régulés codent pour des enzymes de la synthèse de cellulose et des précurseurs de protéines ayant des fonctions liées aux parois cellulaires telles que l'UDP-glucose 6-déshydrogenase,

l'UDP-L-rhamnose synthase, et les extensines riches en proline. La glycolyse, la synthèse des acides organiques et le métabolisme primaire ont été également affectés par les fortes concentrations de CO_2 avec l'augmentation des transcrits comme la phosphoénolpyruvate carboxylase (PEPC), l'oxoglutarate décarboxylase et l'ATP-citrate synthase. La tréhalose phosphate synthase et la tréhalose-6-phosphate impliquées dans le métabolisme du tréhalose, la glucane synthase-like impliquée dans la biosynthèse de la callose, la fructokinase impliquée dans la dégradation du saccharose ont aussi été induites par les concentrations élevées de CO_2.

Les transcrits régulés à la hausse dans la catégorie du métabolisme secondaire sont des gènes ayant des fonctions dans la synthèse des anthocyanines, des lignines et des flavonoïdes, tels que la production du pigment anthocyanine, la leucoanthocyanidin dioxygenase et la phenylalanine ammonia-lyase 1. Des transcrits associés aux transports du glucose-6-phosphate, d'acides aminés et de vésicules sont aussi régulés à la hausse. L'augmentation des transcrits codant pour des protéines de résistance aux maladies, des protéines de choc thermique et des protéines de réponse à la déshydratation indique une réponse active des plantes à l'environnement enrichi en CO_2.

La plupart des gènes régulés à la baisse ont été localisés dans les plastides tandis que les gènes régulés à la hausse étaient beaucoup plus impliqués dans les systèmes membranaires.

De façon générale, l'exposition aux concentrations élevées de CO_2 chez *Arabidopsis* altère l'expression de gènes impliqués dans la défense, le contrôle rédox, le transport, la signalisation, la transcription et le remodelage de la chromatine. Les deux variétés analysées ont perçu les concentrations élevées de CO_2 comme une perturbation métabolique qui a nécessité une augmentation des processus ayant des fonctions dans la consommation ou l'emmagasinage des photoassimilats. Par contre, l'une des variétés présente une meilleure acclimatation qui est associée à sa capacité d'ajuster l'homéostasie et la signalisation des espèces réactives de l'oxygène.

1.3.2 Chez le soya

La réponse du soya (*Glycine max*) aux concentrations élevées de CO_2 au niveau du transcriptome des feuilles en croissance et des feuilles parfaitement développées a été analysée par Ainsworth et collaborateurs (2006) en utilisant la technique des micropuces. Leurs études ont montré que 327 gènes ont une plus forte expression en présence de concentrations élevées de 550 ppm comparées aux concentrations ambiantes de CO_2 de 375 ppm. La plupart de ces gènes ont des rôles dans les fonctions cellulaires telles que le cycle cellulaire, la transcription et la régulation de l'ARN, la synthèse d'ADN et l'organisation cellulaire. Les facteurs de transcription étaient les plus représentés. L'augmentation de l'expression des facteurs de transcription est corrélée aussi avec celle des enzymes impliquées dans la dégradation des protéines. Ceci inclus les protéases spécifiques à l'ubiquitine, les protéinases Cys, et des sous-unités différentes de protéasome. Ces résultats suggèrent que la croissance durant l'exposition aux concentrations élevées de CO_2 accélère le taux de renouvellement des protéines. Les autres gènes dont l'expression est différente en présence de concentrations de CO_2 élevées sont impliqués dans le transport, le métabolisme de l'azote ainsi que des hormones, le métabolisme secondaire (en particulier la biosynthèse de lignine) et le métabolisme du carbone. Les enzymes ß-amylase et exoamylase impliquées dans la dégradation de l'amidon ont été régulées à la hausse. Les niveaux élevés de phosphogucomutase suggèrent que le « pool » de phosphate était plus important chez les feuilles exposées aux concentrations de CO_2 élevées, ce qui est en accord avec l'augmentation de la disponibilité en hexoses à partir de la dégradation de l'amidon. La phosphofructokinase impliquée dans la première étape de la glycolyse, ainsi que toutes les enzymes requises pour la synthèse de phosphoénolpyruvate à partir du glycéraldéhyde-3-P ont été régulées à la hausse. Les concentrations élevées de CO_2

ont induit également les niveaux de transcrits des gènes qui codent pour des enzymes impliquées dans le cycle des acides tricarboxyliques (TCA) comme la pyruvate déshydrogénase ainsi que dans le cycle des pentoses phosphate (OPP) comme la phosphogluconate déshydrogénase. Ces résultats sont en accord avec l'augmentation de la respiration foliaire observée chez plusieurs espèces de plantes (Davey *et al.*, 2004).

1.3.3 Chez le riz

Fukayama et ses collaborateurs (2005) ont comparé le profil d'expression des gènes au niveau des feuilles de riz exposées à une concentration de CO_2 élevée de 68 Pa et des feuilles témoins exposées à une concentration ambiante de CO_2 de 38 Pa. Parmi les 44000 transcrits présents dans leurs analyses par micropuces, 46 gènes sont régulés à la hausse avec une augmentation de plus de 1,5 fois de leur expression par rapport aux feuilles témoins et 35 gènes sont régulés à la baisse avec moins de 0.68 fois. La plupart de ces gènes sont impliqués dans le système de signalisation et dans la régulation de la transcription. Par contre, l'expression des gènes impliqués dans le métabolisme primaire n'a pas été altérée de façon significative. Bien que ces changements soient minimes, les gènes codant pour les enzymes impliquées dans la fixation du carbone (anhydrase carbonique, RUBISCO, phosphoglycérate kinase et glyceraldehyde-3-phosphate déhydrogénase) ont été régulés à la baisse tandis que les gènes codant pour les enzymes de la régénération du RuBP (fructose bisphophate phosphatase, fructose biphosphate aldolase, sédulohéptulose biphosphate phosphatase et phosphoribulokinase) ainsi que les enzymes de la synthèse de l'amidon (ADP-glucose pyrophosphorylase et amidon synthase) ont été régulés à la hausse sous les concentrations élevées de CO_2. Ces résultats suggèrent que des séries de gènes pourraient être co-régulés par les concentrations élevées de CO_2.

1.3.4 Chez le peuplier

Les bases moléculaires de la réponse des plantes face aux concentrations élevées de CO_2 ont été étudiées par Gupta et ses collaborateurs (2005) chez *Populus tremuloïdes*. Le profil d'expression de 4500 EST a été étudié suite à une exposition au CO_2 et/ou à l'ozone. Le traitement au CO_2 a entraîné une augmentation des transcrits des gènes photosynthétiques codant pour les protéines chloroplastiques ribosomales 30S, le photosystème II, ainsi que des gènes de croissance et des gènes de la voie de biosynthèse des auxines. Ceci suggère que l'appareil photosynthétique continue de produire de plus grandes quantités de transcrits chez le peuplier exposé à des concentrations élevées de CO_2. Un autre transcrit qui s'accumule de façon significative est celui de la xyloglucan endotransglycolyase, responsable de l'expansion de la paroi cellulaire. L'expression de ce gène clé est corrélée avec celle de plusieurs autres gènes codant pour des éléments variés du cytosquelette associé à la croissance tels que les sous-unités α et β de la tubuline, et plusieurs protéines qui sont des facteurs de la dépolymérisation de l'actine. Selon les auteurs, l'expression de ces gènes contribuerait à expliquer le fait que les feuilles en croissance dans des conditions de CO_2 élevée soient plus larges que dans les conditions ambiantes telles que rapportées par les études physiologiques.

Les gènes qui présentent une diminution très significative de leur expression suite à l'exposition aux concentrations élevées de CO_2 incluent la protéine membranaire impliquée dans le transport de l'eau, l'aquaporine PIPa2, et la flavonone 3-β-hydrolase impliquée dans le métabolisme des flavonoïdes. D'autres enzymes impliquées dans la défense des plantes face aux stress environnementaux telles que la chalcone flavone isomérase et la cinnamate-4-hydroxylase présentent une diminution de leur expression. La RUBISCO a également été régulée à la baisse, ce qui suggère que l'appareil photosynthétique devient limité à cause du manque de disponibilité de la RUBISCO pour fixer le CO_2, au fur et à mesure que la teneur en CO_2 arrive à

saturation au niveau des feuilles durant l'exposition aux concentrations élevées de CO_2 (Gupta *et al.*, 2005).

1.3.5 Chez le fraisier

Ponce-Valadez *et al.*, (2009) ont étudié l'expression des gènes régulés par les concentrations élevées de CO_2 (20Kpa) durant 48 h au niveau des fruits de deux variétés de fraises. La variété Jewel se distingue par sa propriété à accumuler des quantités élevées d'éthanol et d'acétaldéhyde en réponse aux concentrations élevées de CO_2, contrairement à la variété Cavendish qui n'a en pas la possibilité. Les auteurs ont utilisé comme sondes, près de 12000 EST codant pour 8700 gènes de tomates dans leurs analyses de micropuces pour hybrider les ADNc de fraises. Chez la variété Jewel, 168 transcrits ont été exprimés de façon différentielle entre les deux concentrations de CO_2. Les transcrits identifiés sont impliqués dans le métabolisme primaire (1 gène), secondaire (3 gènes) et des acides nucléiques (10 gènes), les signaux de transduction (14 gènes), la synthèse et la dégradation des protéines (10 et 8 gènes), la transcription (7 gènes), le stress (6 gènes), et le transport (4 gènes). Chez la variété Cavendish, 51 transcrits ont été exprimés de façon différentielle entre les deux concentrations de CO_2. Les transcrits identifiés font partie des mêmes catégories de groupements fonctionnels identifiés chez la variété Jewel à l'exception de celui des métabolites secondaires puisqu'aucun transcrit de cette catégorie n'a été identifié chez Cavendish. De plus, les facteurs de transcription et les gènes impliqués dans la synthèse des protéines sont différents chez les deux variétés (Ponce-Valadez *et al.*, 2009).

1.4 COMMENT LES PLANTES PERÇOIVENT ET RÉPONDENT AUX EFFETS DES BASSES TEMPÉRATURES?

1.4.1 Perception du froid

1.4.1.1 Les membranes

Bien que les détecteurs de température au niveau des plantes n'aient pas encore été identifiés, les travaux actuels suggèrent que les altérations de la fluidité et la rigidité de la membrane plasmique représentent un des premiers événements de la perception des basses températures. Les basses températures entraînent une diminution de la fluidité membranaire alors que l'augmentation de la température permet une meilleure fluidité des membranes (Alonso *et al.,* 1997; Danyluk *et al.,* 1998). Cette hypothèse est supportée par les études qui ont démontré que l'application de certains produits pharmacologiques qui modifient la fluidité membranaire entraînait également un retard de l'expression des gènes de réponse au froid. Par exemple, chez la luzerne (*Medicago sativa*), l'expression du gène *CAS30* connu pour être spécifique à l'acclimatation au froid est bloquée par l'application de l'alcool benzylique (Orvar *et al.,* 2000). Chez *Brassica napus*, l'application de DMSO en absence de traitement au froid a conduit à l'induction du gène *BN115* qui est l'orthologue de *COR15a* chez *Arabidopsis* (Sangwan *et al.,* 2001). L'utilisation des mutants déficients en désaturase acide pour simuler les changements de la rigidité membranaire sans utiliser d'agents pharmacologiques a abouti à des résultats similaires (Vaultier *et al.,* 2006).

1.4.1.2 Photosystème

Les plantes peuvent percevoir les variations de la longueur du jour, de la qualité et de l'intensité lumineuse. La lumière peut aussi être d'une importance

capitale pour la perception de la température car les processus de la photosynthèse sont un des premiers à être affectés par les changements de température. De plus, la capacité des plantes à développer une résistance au gel est associée à la présence de la lumière et de l'activité photosynthétique durant l'acclimatation au froid (Gray *et al.*, 1997).

Les réactions photochimiques de la photosynthèse permettent de capter l'énergie lumineuse par l'entremise des deux photosystèmes et de la transformer sous forme de potentiel rédox. Les réactions biochimiques convertissent ce potentiel énergétique rédox en pouvoir réducteur stable, en l'occurrence le NADPH. Les réactions photochimiques sont très rapides et dépendantes de la lumière, contrairement aux réactions biochimiques qui sont plus lentes et diminuent avec la baisse des températures. La diminution des réactions biochimiques lors des basses températures crée un déséquilibre entre l'énergie absorbée et celle transférée. Les électrons provenant du photosystème I sont transférés à l'oxygène, ce qui crée des espèces réactives de l'oxygène (ROS), du peroxyde d'hydrogène (H_2O_2) et des radicaux hydroxyles (Winfield *et al.*, 2010).

Les espèces réactives de l'oxygène sont particulièrement intéressantes car elles peuvent jouer deux rôles durant le stress associé au froid. Elles sont un des principaux responsables des dommages au niveau de l'ADN, des protéines et lipides. De plus, les variétés de plantes résistantes au froid sont connues pour avoir des systèmes anti-oxydants beaucoup plus efficaces que les espèces sensibles aux basses températures (Suzuki and Mittler, 2006). Les ROS jouent aussi un rôle de médiateur du système de transduction de signaux associé aux stress abiotiques. La production de ROS induite par la lumière joue un rôle central dans la perception de la basse température (Suzuki and Mittler, 2006). L'habilité des plantes à développer la résistance au froid est associée à la présence de la lumière et de l'activité

photosynthétique durant la période d'acclimatation au froid. En effet, il a été démontré que le traitement au froid en présence de lumière augmente de deux fois le nombre de gènes répondant au froid par rapport au nombre de gènes induits à l'obscurité dans les mêmes conditions (Soitamo *et al.,* 2008). Chez la fétuque des prés (*Festuca pratensis*), environ 50% des protéines dont l'abondance change durant l'acclimatation au froid sont impliquées dans la photosynthèse (Kosmala *et al.,* 2009).

Le chloroplaste utilise la lumière comme source d'énergie et réagit aux variations de l'intensité lumineuse en adaptant le métabolisme à l'état rédox de la chaîne de transport des électrons. L'augmentation de la pression d'excitation du photosystème II est un des premiers stimuli qui induit l'expression des gènes répondant au froid (Gray *et al.,* 1997; Ndong *et al.,* 2001). À l'opposé, l'exposition des plantes au froid en absence de lumière réduit l'induction de plusieurs gènes de réponse au froid (Crosatti *et al.,* 1999; Kobayashi *et al.,* 2004). Chez l'orge Albina, les mutants qui sont incapables de développer correctement leurs chloroplastes présentent une diminution de l'expression des gènes de réponse au froid (Svensson *et al.,* 2006).

1.4.1.3 Composition du nucléosome

Récemment Kumar et Wigge (2010) ont montré que l'histone H2AZ joue un rôle important durant la perception de la température chez *Arabidopsis* et devrait être responsable de l'expression coordonnée de gènes sensibles à la température. Il a été suggéré que H2AZ est présente dans la chromatine, immédiatement en aval des promoteurs des gènes de réponse à la température et ceci crée une barrière physique à l'ARN polymérase II et l'expression des gènes. Avec l'augmentation de la température, H2AZ est enlevée de ces sites permettant l'expression des gènes impliqués. En ce qui concerne les gènes exprimés par les basses températures, il a été proposé que l'occupation des sites par H2AZ empêche les répresseurs de se fixer. Les

auteurs ont montré l'implication de la protéine H2AZ dans la régulation d'expression des gènes chez la levure, suggérant que ceci est un mécanisme conservé dans l'évolution (Kumar and Wigge, 2010).

1.4.1.4 Le calcium comme messager secondaire

Il y a beaucoup de preuves de l'implication du calcium comme second messager dans le système de signalisation cellulaire relié aux basses températures. Chez *Arabidopsis* et la luzerne, le niveau de calcium cytoplasmique augmente rapidement en réponse aux basses températures (Knight *et al.,* 1984; Monroy *et al.,* 1997). L'influx de calcium dans le cytosol est une des premières réponses suite à la détection du changement de température au niveau de la cellule végétale. Cet influx est médié par les canaux de calcium et induite par les espèces réactives d'oxygène reliées au stress. Les chélateurs de calcium et les bloqueurs des canaux ioniques tels que le lanthane inhibent l'influx de calcium et causent une diminution de l'expression du gène *cas15* inductible par le froid chez la luzerne (Monroy *et al.,* 1997). Cette inhibition bloque la capacité de la luzerne à s'acclimater au froid. Par contre, la nature des canaux qui sont responsables de l'influx de calcium durant l'acclimatation au froid chez *Arabidopsis* et la luzerne n'a pas été déterminée. Les étapes entre l'influx de calcium et l'activation de l'expression des gènes et de l'acclimatation au froid sont relayées par l'action des protéines kinases et des phosphatases.

Le froid est associé à une accumulation de calcium intracellulaire et cette augmentation est perçue par diverses protéines de liaison du calcium. Les trois principales protéines de liaison au calcium sont les Cam (calmodulin-like), les CDPK (calcium dependent protein kinases) et les CBls (Calcineurin b-like proteins). Ces protéines contiennent un domaine conservé appelé EF qui permet de lier le calcium (Yang *et al.,* 2004). La liaison du calcium aux protéines entraîne un changement de conformation de ces dernières qui leur permet d'interagir et de réguler leurs protéines

cibles (DeFalco *et al.,* 2010). En retour, ces effecteurs initient une série d'évènements (phophorylation et déphosphorylation) qui résultent en une reprogrammation de l'expression des gènes observée durant l'acclimatation au froid.

1.4.2 Acclimatation au froid, tolérance aux basses températures et vernalisation

Dans les régions tempérées, la baisse de température et de lumière entre l'automne et l'hiver est un évènement clé qui permet aux plantes d'anticiper le changement de saison et se préparer contre les changements environnementaux. Durant cette période, certaines plantes ont la capacité d'induire ou d'augmenter leurs mécanismes de tolérance au froid. Le blé et les céréales telles que le seigle et l'orge peuvent s'acclimater à des conditions climatiques diverses grâce à leur large variabilité génétique. Ainsi le blé offre une large gamme de variétés tolérantes au froid et au gel. Par contre, le riz, le maïs et la tomate sont très sensibles au froid et ne sont pas capables d'endurer le gel (Fowler and Tyler, 1981). Les céréales d'hiver sont capables de tolérer des températures suboptimales alors que les variétés plus sensibles n'en sont pas capables. Cette capacité de résistance aux basses températures n'est pas un caractère constitutif et requiert une période préalable d'exposition au froid. Cette période d'exposition au froid permet à la plante de s'acclimater. Le blé d'hiver cv Norstar peut tolérer des températures de -20 °C lorsqu'il est au préalable exposé au froid (5 °C). Lorsqu'il n'est pas acclimaté, il ne survit pas à des températures plus basses que -5 °C (Monroy *et al.,* 2007). Plusieurs processus d'ordre moléculaire, physiologique et génétique sont à l'origine de cette acclimatation au froid et la tolérance aux basses températures (Houde *et al.,* 1992; Danyluk *et al.,* 1998; Badawi *et al.,* 2007).

La température est un signal clé qui contrôle la transition du stade végétatif au stade reproductif assurant la floraison des plantes lorsque les conditions saisonnières sont idéales. La vernalisation est le processus par lequel l'exposition prolongée des

plantes à des températures froides peut accélérer la compétence de ces dernières à fleurir. La capacité à promouvoir la floraison dépend également d'autres facteurs environnementaux tels que la photopériode. Les céréales d'hiver ont besoin de vernaliser afin d'initier leur floraison, contrairement aux variétés de printemps qui ne nécessitent pas cette exposition préalable pour fleurir (Kane *et al.,* 2005). Bien que l'acclimatation au froid et la vernalisation soient des réponses aux basses températures, la durée de l'exposition au froid qui initie ces réponses est différente. Des expositions courtes de 1 à 2 jours sont suffisantes pour induire l'acclimatation au froid alors que la vernalisation requiert une période d'exposition au froid beaucoup plus longue (Sung and Amasino, 2005).

La plupart des céréales ont des degrés de tolérance aux basses températures constitutifs. L'acclimatation au froid et l'acquisition de la tolérance au gel, par contre, requièrent l'orchestration de mécanismes physiologiques, moléculaires et biochimiques. Ces mécanismes sont en partie possibles grâce à l'expression différentielle de beaucoup de gènes. Ces gènes sont induits par le froid et d'autres stress associés au froid (Guy *et al.,* 1985). Plusieurs gènes régulés par le froid ont été identifiés par des analyses du transcriptome. Chez *Arabidopsis*, par exemple, des centaines de transcrits ont été rapportés comme répondant au froid (Chen *et al.,* 2002). Chez le blé, Monroy et collaborateurs (2007) ont identifié 450 gènes qui sont régulés par le froid. Bien que ces gènes aient été sélectionnés sur la base de leur réponse au froid, leur fonction spécifique et leur rôle durant l'acclimatation au froid n'ont pas tous été déterminés.

1.4.3 Changements globaux des transcrits suite à l'exposition au froid

Il est bien établi que l'exposition des plantes au froid mène à des changements au niveau de l'expression de certains gènes (Guy *et al.,* 1985). Durant la dernière décennie, l'analyse du transcriptome grâce à la technologie des micropuces révèle

qu'environ 4% des gènes chez *Arabidopsis* répondent à une exposition courte au froid de 24 h et 20% des gènes lors d'une exposition plus longue de plus de 14 jours (Hannah *et al.,* 2005; Lee *et al.,* 2005). Chez le blé, 8% des 5740 cDNA enrichis en gènes régulateurs et de signalisation analysés à l'aide de micropuces montrent un changement de leur expression lors d'une exposition au froid (Monroy *et al.,* 2007). Toujours chez le blé, Winfield et collaborateurs (2010) en utilisant les micropuces d'affymetrix (62000 sondes) ont démontré que le nombre de transcrits changeant suite à une exposition de 2 jours au froid pour trois variétés de blé (Harnesk, Paragon et Solstice) était de 2.85, 3.46 et 2.3%, respectivement.

1.4.4 Les gènes impliqués dans la réponse au froid

Dans la nature, le froid précède le gel et permet d'induire des changements moléculaires au niveau des cellules chez les espèces de plantes tolérantes. Les basses températures affectent le captage de l'eau et des nutriments, la fluidité membranaire ainsi que la conformation des acides nucléiques et influencent dramatiquement le métabolisme cellulaire soit directement par réduction des réactions biochimiques ou indirectement à travers la reprogrammation de l'expression des gènes. Un nombre élevé de gènes induits par les basses températures ont été identifiés et caractérisés. Ces gènes comprennent les LEA (late embryogenesis abundant), les DHN (déhydrines), les RAB (répondant à l'acide abscissique), les LT (réponse aux basses températures), les AP2/ CBFs et les COR (répondant au froid). Le niveau d'expression de plusieurs gènes de réponse au froid (COR) est fortement associé avec la tolérance au gel. Par exemple, la surexpression du gène *WCS19* du blé permet à Arabidopsis de mieux tolérer le froid (NDong *et al.,* 2002).

Parmi ces produits de gènes associés à la tolérance au gel, plusieurs protéines structurales telles que les protéines chaperonnes, les osmoprotectants, les protéines de liaison à la glace sont directement impliquées dans la protection des plantes contre le

stress. D'autres sont des régulateurs de gènes tels que les facteurs de transcription, des protéines kinases et des enzymes impliquées dans la synthèse des hormones végétales. Les facteurs de transcription et les gènes qui les contrôlent sont appelés régulons. Un des régulons les plus étudiés durant dans les réponses au froid est celui des CBFs.

1.4.4.1 Les facteurs de transcription CBFs et leurs régulons

Les facteurs de transcription CBFs font partie de la famille des protéines AP2/EREBP liant l'ADN et qui reconnaissent l'élément CRT/DRE (C-repeat/dehydration-responsive element). Ces éléments ayant un domaine de 5 paires de bases conservées CCGAC, sont présents dans la région promotrice de plusieurs des gènes de réponse au froid et à la sécheresse. Les CBF sont induits rapidement, seulement 15 min après l'exposition au froid, et 2 h plus tard il est possible de voir l'induction des gènes du froid ayant le domaine de régulation CRT/DRE (Gilmour *et al.,* 1998; Badawi *et al.,* 2007). La surexpression de CBF3 dans des plantes transgéniques d'*Arabidopsis* entraîne une induction de l'expression des gènes *COR* et une augmentation de la tolérance au gel en absence de stimulus de basses températures (Gilmour *et al.,* 2000). Cette plus grande tolérance aux basses températures est associée à l'accumulation d'osmoprotectants tels que la proline et le stachyose au niveau des plantes surexprimant *CBF3*, et ce, sans acclimatation préalable au froid. Il a donc été suggéré que les gènes *CBFs* agissent en intégrant l'activation de plusieurs composants de la réponse à l'acclimatation au froid. Les *CBFs* ont été caractérisés chez Arabidopsis, le riz, et le blé (Cook *et al.,* 2004; Ito *et al.,* 2006; Badawi *et al.,* 2007). Chez le blé, il y a plus de 25 gènes *CBFs* différents localisés dans le long bras du chromosome 5. Les études d'expression ont montré que 6 membres du groupe des *CBFs* (IIIc, IIId, IVa, IVb, IVc et IVd) sont exprimés uniquement chez la famille des *Pooideae* comprenant le blé, l'orge et le seigle. De plus, l'induction et le niveau d'expression des *CBFs* IIId, IVa, IVb, IVc et IVd par les

basses températures sont beaucoup plus élevés chez les cultivars d'hiver comparés à ceux de printemps (Badawi *et al.,* 2007; Sutton *et al.,* 2009). Cette expression plus élevée chez les cultivars d'hiver explique en partie la plus grande capacité de tolérance des variétés d'hiver et est possiblement la base de la variabilité génétique au niveau de la tolérance au gel dans la famille des *Pooideae.*

Les *CBFs* sont régulés par deux voies de signalisation, l'une reliée à la température et l'autre à la lumière. Badawi et collaborateurs (2007) ont montré que certains *CBFs* sont régulés par la lumière. De plus, il a été rapporté que la qualité du signal de la lumière (mesurée par le ratio de lumière rouge/rouge lointain) médiée à travers les phytochromes et les cryptochromes, régule l'expression des *CBFs* (Franklin, 2009).

Chez Arabidopsis, le gène *ICE1* (inducteur de CBF) agit en aval et contrôle l'expression du régulon CBF. Le produit de *ICE1* est un facteur de transcription du type MYC ayant le motif hélice-boucle-hélice qui reconnaît et lie les sites MYC ou appelés aussi cassette ICE au niveau du promoteur de *CBF3* et induit son expression. Les études chez le mutant *ice1* ont montré que ce dernier était incapable de s'acclimater au froid. De plus, ces mutants sont très sensibles au froid et l'induction de *CBF3* observée généralement durant l'exposition au froid fait défaut. La surexpression constitutive de *ICE1* chez *Arabidopsis* a mené à des niveaux élevés d'expression des *CBF2, CBF3* et des gènes *COR* durant l'acclimatation au froid et a permis une meilleure tolérance au gel. *ICE1* est un gène exprimé de façon constitutive et des modifications post-traductionnelles de sa protéine induites par le froid sont requises pour l'expression de CBF (Chinnusamy *et al.,* 2007). La surexpression de *ICE1* chez le riz a permis une meilleure tolérance au froid de cette espèce sensible (Xiang *et al.,* 2008). Bien que les régulons CBF semblent être parmi les plus importants impliqués dans l'acclimatation au froid, ils ne sont pas les seuls

Chez *Arabidopsis*, seulement 12% des gènes induits par le froid sont associés au régulon des CBFs (Chinnusamy *et al.,* 2007). Chez le blé, plus d'un tiers des gènes induits par le froid ne sont pas assujetis à la régulation par les CBFs (Monroy *et al.,* 1997).

1.4.4.2 Les facteurs de transcription WRKY

Les protéines WRKY sont codées par une large famille de gènes. Chez le riz, plus d'une centaine de gènes ont été identifiés et 74 chez Arabidopsis (Berri *et al.,* 2009). Ils sont retrouvés presque exclusivement chez les plantes et les algues vertes (Eulgem *et al.,* 2000). Ils sont caractérisés par un ou deux domaines conservés de 60 acides aminés appelés WRKY qui contiennent un motif en doigt de zinc permettant de lier l'ADN. Le domaine WRKY lie spécifiquement les séquences au niveau du motif W [(C/T)TGAC(C/T)] sur le promoteur des gènes cibles. Récemment, une étude chez le blé suggère que les facteurs de transcription WRKY seraient impliqués dans la tolérance au froid (cold hardening) (Talanova *et al.,* 2009). Par ailleurs, plusieurs gènes de défense contre l'attaque des pathogènes présentent une surreprésentation des motifs W au niveau de leur promoteur. Les WRKY eux-mêmes en possèdent au niveau de leur promoteur et sont ainsi autorégulés.

1.4.5 Les effecteurs en réponse au froid et au gel

Beaucoup de molécules ayant des propriétés d'osmoprotectants s'accumulent chez les plantes durant l'exposition au froid. Ces métabolites ont des rôles importants dans la protection contre les dommages causés par le gel. La synthèse d'antioxydants, l'accumulation de sucres dans l'espace apoplastique, ainsi que l'induction de

molécules chaperonnes permettent de stabiliser les membranes durant le froid (Guy et Li 1998; Livingston et Henson 1998).

Les molécules effectrices synthétisées en réponse au froid ou au gel ont des fonctions diverses. Les osmoprotectants tels que la proline et les sucres simples permettent de stabiliser les membranes au niveau des lipides et des protéines. Les chaperonnes protègent les protéines contre les changements structuraux induits par le froid. Il y a aussi des inhibiteurs de la formation de glace, ainsi que des enzymes photosynthétiques impliquées dans l'homéostasie entre les photosystèmes I et II et les réactions biochimiques du Cycle de Calvin. Les enzymes de la respiration ainsi que les antioxydants qui éliminent les ROS sont également impliqués dans la réponse au froid (Winfield *et al.,* 2010).

1.4.5.1 Les déhydrines

Les conséquences majeures du stress associé au froid sont la déshydratation et le stress osmotique associé à ce dernier. Plusieurs gènes de réponse au froid codent pour des déhydrines. Les déhydrines sont un groupe distinct des protéines LEA (late embryogenesis abundant) qui contiennent un motif d'acides aminés riche en lysine appelé le segment K (Allagulova *et al.,* 2003; Kosová *et al.,* 2007). Les déhydrines ont la particularité d'être très hydrophiles, solubles même après ébullition et riches en glycine et acides aminés polaires. Leur expression est induite par plusieurs facteurs environnementaux tels que la chaleur, la déshydratation et la salinité (Allagulova *et al.,* 2003). Le froid et le gel causent un stress osmotique et il a été démontré que l'induction et l'accumulation des déhydrines est un important élément dans l'acclimatation au froid des céréales d'hiver (Danyluk *et al.,* 1998). Ils peuvent jouer le rôle d'émulsifiants ou de chaperonnes afin de protéger les protéines membranaires contre les dommages de la déshydratation. Ces protéines ont également la capacité de lier les membranes mitochondriales (Borovskii *et al.,* 2005).

Une famille bien connue parmi les gènes de réponse au froid est celle des gènes *WCS120* (wheat cold specific). Cette famille est composée de 5 gènes qui codent pour des protéines de 200, 180, 66, 50 et 40 kDa. Ces protéines marqueurs de l'acclimatation au froid s'accumulent en plus grande quantité dans les variétés d'hiver comparées à celles de printemps. Les protéines des gènes *WCS120* régulés par le froid et impliqués dans la protection contre la déshydratation ont des propriétés cryoprotectrices aussi efficaces que le saccharose et l'albumine du sérum bovin (BSA) pour la protection de la lactate déshydrogénase (LDH) contre la dénaturation causée par le gel (Houde *et al.,* 1992).

1.4.5.2 Les espèces réactives de l'oxygène

Le stress associé aux basses températures a un effet dramatique sur le métabolisme des plantes en créant une perturbation de l'homéostasie cellulaire et le découplage des processus physiologiques, entraînant ainsi la formation accélérée d'espèces réactives de l'oxygène (Suzuki and Mittler, 2006). Ces molécules sont des radicaux toxiques qui causent des dommages aux protéines, à l'ADN et aux lipides, ce qui perturbe les fonctions cellulaires pouvant mener à la mort cellulaire. Le chloroplaste est très sensible aux dommages des espèces réactives d'oxygène qui sont générées par la réaction de l'oxygène chloroplastique et des électrons qui s'échappent du système de la chaîne de transport (Foyer *et al.,* 1994). Les cellules possèdent des antioxydants capables d'interrompre la cascade incontrôlée de l'oxydation des organelles cellulaires. Le stress oxydatif résulte d'un déséquilibre entre la formation de ROS et leur neutralisation par les antioxydants. L'acclimatation au froid est associée à l'accumulation d'enzymes et de métabolites capables de se débarrasser des ROS (Tao *et al.,* 1998). Ces enzymes, combinées avec d'autres antioxydants, forment un système localisé au niveau du photosystème II qui fournit une protection contre la toxicité associée aux espèces réactives de l'oxygène. Ce système est très dépendant de l'eau à cause du flux d'électrons et est appelé cycle de l'eau (Asada, 1999). La

première enzyme de ce système est la superoxyde dismutase qui scinde le superoxyde en peroxyde et en oxygène. Ensuite, l'ascorbate peroxydase permet de détoxifier le peroxyde en eau grâce à l'utilisation de l'ascorbate comme réducteur. La glutathione réductase quant à elle réduit le métabolite rédox glutathion qui sert d'antioxydant.

1.4.5.3 Les flavonoïdes

Les flavonoïdes sont des métabolites secondaires dérivés de la phénylalanine et du métabolisme de l'acétate qui effectuent une variété de fonctions essentielles chez les plantes supérieures telles que le rôle d'antioxydant. Les enzymes chalcone synthase et chalcone isomérase sont des enzymes clés de la biosynthèse des flavonoïdes et sont régulées par le froid. Les études de Winfield et collaborateurs (2010) ont montré que le transcrit de l'enzyme chalcone synthase est 20 fois plus exprimé dans les variétés d'hiver comparées aux variétés de printemps. De plus, les transcrits de chalcone synthase augmentent de 20 fois et 3 fois chez deux variétés de blé d'hiver acclimatées au froid comparées aux plantes non acclimatées. Une autre protéine potentielle, la 7-O-glycosyltransferase a montré un profil d'accumulation similaire, tandis que le transcrit d'une protéine chalcone isomérase diminue au niveau des feuilles des variétés d'hiver. Ces profils d'expression sont des indications de l'implication de la voie de signalisation des flavonoïdes dans la réponse associée au stress froid.

1.4.5.4 Les protéines antigel

Une fois acclimatés, les cultivars d'hiver sont capables de tolérer une exposition aux basses températures jusqu'à -20 °C chez le blé cv Norstar et -25 °C chez le seigle (Ouellet *et al.*, 1998; Monroy *et al.*, 2007). Un des premiers sites endommagés par le gel est la membrane plasmique. La tolérance au froid est

étroitement liée aux mécanismes par lesquels les cellules végétales évitent les dommages aux membranes cellulaires. Une bonne partie de cette tolérance dépend de la capacité à éviter la formation de cristaux de glace à l'intérieur et à l'extérieur de la cellule. Le gel à l'extérieur des cellules crée une déshydratation à cause du mouvement de l'eau qui quitte le cytoplasme pour former des cristaux de glace à l'extérieur de la cellule. La formation des cristaux de glace crée un stress mécanique qui se traduit par une déformation des membranes cellulaires qui finissent par se rompre et perdre leur perméabilité. Une des fonctions de l'acclimatation au froid est de stabiliser les membranes contre les dommages du gel. Les protéines antigel permettent de retarder et de limiter la formation de cristaux de glace. Les osmoprotectants peuvent aussi protéger les membranes et les protéines des effets de la déshydratation et de la modification de la composition des membranes cellulaires.

1.4.5.5 Les chitinases, glucanases, et thaumatines

Lorsque la température diminue aux environs du point de congélation, la formation de glace est initiée au niveau des vaisseaux du xylème à cause de la concentration de solutés qui y est plus faible (Ristic and Ashworth, 1994). Durant l'acclimatation au froid, les plantes tolérantes au gel accumulent des protéines antigel par anticipation de l'arrivée des conditions hivernales. Ces protéines qui s'accumulent principalement au niveau de l'apoplaste (xylème, paroi cellulaire et espace intercellulaire) ont des caractéristiques similaires aux protéines PR qui sont régulées par les pathogènes. Elles comprennent les chitinases, les glucanases et les thaumatines (Antikainen *et al.*, 1996; Pihakaski-Maunsbach *et al.*, 1996; Bishop *et al.*, 2000). Ces trois types de protéines appartiennent à de larges familles de gènes impliqués dans la réponse aux stress biotiques et abiotiques (Karlsson and Stenlid, 2008).

Les protéines PR sont relarguées dans l'apoplaste et forment des complexes avec des composants variés (Yaish *et al.*, 2006). Elles peuvent se fixer aux cristaux de glace et empêcher leur croissance. Elles peuvent aussi inhiber la recristallisation de la glace qui survient lorsque les températures fluctuent aux environs du point de congélation, ce qui résulte en une migration des molécules d'eau des petits cristaux aux grands cristaux de glace (Monroy *et al.*, 1997). Bien que les chitinases et glucanases n'aient pas de domaine de liaison à la glace, elles se distinguent des autres protéines PR par leur capacité à adopter une structure tridimensionnelle avec une surface pouvant lier la glace (IBS). Elles peuvent donc se lier aux cristaux de glace grâce à des liaisons hydrogènes et des interactions de Van der Waals, inhibant ainsi leur croissance et la recristallisation (Yeh *et al.*, 2000; Yaish *et al.*, 2006).

1.4.5.6 Protéines inhibitrices de la recristallisation de la glace (IRI)

Les deux protéines inhibitrices de la recristallisation de la glace, TaIRI-1 et TaIRI-2, appartiennent à une classe de protéines liant la glace spécifique à la famille des *Pooideae* incluant le blé, l'orge et le seigle (Tremblay *et al.*, 2005). Ces protéines contiennent une courte partie N-terminale riche en leucine qui a une homologie avec les récepteurs kinases et un domaine C-terminal homologue au domaine de liaison de la glace des protéines antigel (Sandve *et al.*, 2008). Les teneurs des deux transcrits TaIRI-1 et TaIRI-2 augmentent suite à la diminution graduelle de la température et à une courte exposition au froid. La protéine TaIRI-2 s'accumule beaucoup plus dans les variétés d'hiver comparées à celles de printemps (Winfield *et al.*, 2010).

1.4.5.7 Les sucres simples

Il est établi depuis fort longtemps que l'accumulation des solutés compatibles tels que les osmoprotectants organiques au niveau du cytoplasme contribuent à la survie au gel. Cette survie passe par l'entremise de la réduction du taux et de

l'étendue de déshydratation, de la séquestration des ions toxiques et/ou de la protection des macromolécules contre la dénaturation induite par la déshydratation (Steponkus, 1984). Les carbohydrates sont reconnus comme jouant un rôle important dans la tolérance au gel et l'accumulation des sucres simples, tel que le tréhalose, le raffinose et le saccharose est corrélée avec une augmentation de la tolérance au gel. L'accumulation de ces sucres simples permet de stabiliser les membranes afin de mieux tolérer les dommages associés au gel (Wanner and Junttila, 1999; Pennycooke *et al.,* 2003; Kaplan *et al.,* 2007).

Chez *Arabidopsis* et pétunia, le raffinose s'accumule durant l'acclimatation au froid (Wanner and Junttila, 1999; Pennycooke *et al.,* 2003). Ce trisaccharide s'accumule suite à une répression de l'enzyme α-galactosidase responsable de sa coupure. Dans leurs expériences de cinétique d'exposition au froid chez le blé, Winfield et collaborateurs (2010) ont observé une accumulation de galactinol synthase (GolS), la première enzyme dans le réseau de signalisation qui mène à la synthèse de raffinose (Taji *et al.,* 2002). L'enzyme GolS est impliquée dans la partition du carbone entre le saccharose et le raffinose, un processus important dans la production de sucres simples comme osmoprotectants. Il a aussi été rapporté que la synthèse de Gols stimulée par le froid est contrôlée par le facteur de transcription DREB1a (CBF3). De plus, le galactinol et le raffinose ont la capacité d'éliminer les radicaux hydroxyles (Taji *et al.,* 2002; Maruyama *et al.,* 2009).

1.4.5.8 Les annexines

Les annexines appartiennent à la famille des protéines liant le calcium. Elles sont très solubles et contiennent une région de liaison au calcium très conservée et un domaine N-terminal variable. La caractéristique de ces protéines est qu'elles peuvent se lier aux phospholipides membranaires de façon réversible et dépendante du calcium. Elles sont principalement cytosoliques mais peuvent s'attacher ou s'insérer

dans la membrane plasmique (Talukdar *et al.,* 2009). Les annexines ont la propriété d'agir comme des transporteurs d'ions et servent donc de canal ionique au niveau de la membrane plasmique. Elles sont également impliquées dans le système de transduction impliquant le calcium cytosolique libre et les espèces réactives d'oxygène (Mortimer *et al.,* 2008; Laohavisit and Davies, 2009).

1.4.5.9 Les protéines germines

Les protéines germines et germin-like (GLP) appartiennent à la famille des cupines qui possèdent un feuillet ß impliqué dans la liaison aux métaux (Zimmermann *et al.,* 2006). Elles jouent un rôle dans la régulation du calcium, du métabolisme de l'oxalate et de la réponse aux pathogènes. Les vraies protéines germines ont une activité oxalate oxydase et sont retrouvées chez les céréales. Les GLPs, par contre, sont des groupes de protéines plus diversifiées et on les retrouve chez les monocotylédones, les dicotylédones, les gymnospermes et les mousses. Le terme GLP s'applique aux protéines ayant un motif germine mais dont la fonction n'est pas celle d'une oxalate oxydase. Toutefois, on ne connaît pas la fonction enzymatique, s'il y en a une. A peu près les deux tiers des protéines germines identifiées présentent une réponse à des stress abiotiques et biotiques variés. Elles sont toutes des glycoprotéines associées à la matrice extracellulaire et ont soit une activité enzymatique (oxalate oxydase ou superoxide dismutase), soit ce sont des protéines structurales ou des récepteurs (Davidson *et al.,* 2009).

1.5 EFFETS DE L'AUGMENTATION DE LA CONCENTRATION DE CO_2 SUR LES RÉPONSES DES PLANTES AU FROID

Peu d'études se sont focalisées sur l'impact de la forte concentration de CO_2 sur la réponse des plantes face aux stress environnementaux, particulièrement le froid.

Parmi ces études, menées chez différentes espèces de plantes, beaucoup de contradictions ont été rapportées.

Des expériences menées chez la luzerne (*Medicago sativa*) ont montré que les plantes acclimatées à 400 $\mu mol.mol^{-1}$ de CO_2 étaient plus tolérantes au froid que celles maintenues à 800 $\mu mol.mol^{-1}$.

Par ailleurs, l'enrichissement en CO_2 a mené à des dommages de congélation plus sévères au niveau des feuilles d'*Eucalyptus paucififlora,* de *Ginko biloba,* et d'une espèce herbacée tempérée (Terry *et al.,* 2000; Obrist *et al.,* 2001). Par contre, il augmente la tolérance au froid chez *Betula alleghaniensis* et chez *Picea mariana* (Wayne *et al.,* 1998; Bigras and Bertrand, 2006).

Les études chez *Picea abies* ont montré qu'il n'y a aucun effet de la concentration de CO_2 sur la tolérance au froid (Dalen and Johnsen, 2004). Il a été montré que la concentration élevée de CO_2, en stimulant la croissance durant l'acclimatation au froid, peut prédisposer les plantes aux dommages du froid (Margolis and Vézina, 1990).

A l'opposé, l'accumulation d'hydrates de carbone causée par la forte concentration de CO_2 peut promouvoir une meilleure congélation grâce à une augmentation des taux de sucres protecteurs tels que le stachyose, le saccharose et le raffinose, qui sont étroitement liés au développement de la tolérance au froid chez la luzerne (Castonguay *et al.,* 1995). Les concentrations élevées de CO_2 augmentent les sucres totaux non structuraux mais elles diminuent les concentrations d'azote dans les tissus des plantes (Skinner *et al.,* 1999). Les études de Dhont *et al.,* (2006) ont montré que les niveaux d'azote endogène qui s'accumulent dans les racines jouent un rôle important durant le passage à l'hiver de la luzerne. Donc les facteurs qui diminuent les réserves d'azote tels que la défoliation ou les concentrations élevées de CO_2

pourraient diminuer la capacité de la luzerne à résister aux stress environnementaux durant l'hiver (Dhont *et al.,* 2003).

1.6 PROBLÉMATIQUE

Les études portant sur les effets de l'augmentation de la concentration de CO_2 sur la physiologie et le développement des plantes ont montré qu'à court terme, les plantes de type C3 perçoivent et répondent à l'augmentation de la concentration de CO_2 à travers des effets directement reliés à l'augmentation de la carboxylation par la RUBISCO et la diminution de l'ouverture des stomates. A l'opposé une exposition à long terme des plantes aux concentrations élevées de CO_2 peut résulter en une acclimatation physiologique impliquant une diminution dans leur capacité d'assimilation du CO_2 (Long *et al.,* 2004). Des études chez *Arabidopsis* ont montré que les concentrations élevées de CO_2 peuvent modifier les substances chimiques induites tels que les glucosinolates lors de l'attaque par les herbivores ainsi que les trichomes impliqués dans le système de défense structurel. Par contre, ces réponses aux concentrations élevées de CO_2 ne sont pas observées chez tous les génotypes (Bidart-Bouzat *et al.,* 2005). Les bases moléculaires reliées aux processus physiologiques de la réponse aux concentrations élevées de CO_2 n'ont été étudiées à grande échelle que chez quelques espèces telles que le soya, le riz, le peuplier, *Arabidopsis* et le fraisier (Taylor *et al.,* 2005; Ainsworth *et al.,* 2006; Li *et al.,* 2008; Fukayama *et al.,* 2009; Ponce-Valadez *et al.,* 2009). Le profil d'expression des gènes régulés par les concentrations élevées de CO_2 dans ces études présente des patrons différents à cause des espèces et des conditions expérimentales variées. Ces résultats indiquent par ailleurs que la réponse aux concentrations élevées de CO_2 dépend du bagage génétique des plantes et il est donc difficile de trouver une réponse générale univoque.

A ce jour, il n'y a pas d'étude à grande échelle sur l'expression des gènes chez le blé en condition de teneurs en CO_2 élevées, de même que pour l'effet combiné du CO_2 et

du froid. Avec plus de 13 millions d'hectares de terres cultivées, le blé est la culture arable la plus importante au Canada. Depuis les débuts de la culture du blé vers 1605, le blé ne cesse de s'adapter au climat canadien. Grâce aux nombreux croisements entrepris depuis lors, les cultivateurs ont réussi à sélectionner des variétés de blé qui survivent aux hivers canadiens rigoureux , au moins dans le sud du pays, et qui mûrissent très vite durant la courte période végétative. Ces variétés ont d'excellents rendements et sont dotées de très grandes qualités meunières et boulangères. Cette capacité de survie aux basses températures est permise par les processus d'acclimatation et de tolérance au gel durant lesquels des changements d'ordre moléculaire, physiologique, et génétique sont observés chez plusieurs céréales (Thomashow, 1998). Par exemple, le blé d'hiver cv Clair peut tolérer des températures basses jusqu' à -20°C lorsqu'il est préalablement acclimaté au froid (5°C), alors qu'il meurt à des températures de -5°C lorsqu'il n'est pas acclimaté (Monroy *et al.*,. 2007).

Plusieurs études ont démontré que l'exposition à un stress préalable peut conférer ou diminuer la résistance face à un autre stress (Rao *et al.,* 1995; Polle *et al.,* 1997; Pritchard *et al.,* 2000; Schwanz and Polle, 2001; Di Toppi *et al.,* 2002; Gillespie *et al.,* 2011). Bien que les concentrations élevées de CO_2 stimulent la photosynthèse et entraînent une augmentation de la production chez plusieurs espèces, l'augmentation de la température peut diminuer ces effets (Morgan *et al.,* 2011). La combinaison des concentrations élevées de CO_2 et l'augmentation de la température seraient moins favorables aux plantes de type C3 qu'à celles de types C4 (Morgan *et al.,* 2011). Par ailleurs, une augmentation de température de croissance de 6°C entraîne une diminution de la production du blé d'hiver cv US de 30% tandis qu'une diminution de température du même ordre entraîne une augmentation de 37% (Tonkaz *et al.,* 2010). L'augmentation de plus de 25% du rendement de blé dans les zones nordiques du Mexique est attribuée à la diminution de la température nocturne (Lobell *et al.,* 2005). Dans une région nordique de l'Inde d'autres études ont montré que la hausse

de température entraînait une baisse de la production de blé malgré les effets bénéfiques des concentrations élevées de CO_2 (Lal *et al.,* 1998)

1.7 OBJECTIFS

L'objectif global de notre travail est de comprendre les bases physiologiques et moléculaires des réponses du blé durant la croissance et le développement en concentrations élevées de CO_2 et de l'influence du froid.

Le premier objectif a consisté à délimiter les contributions potentielles de la plasticité aux niveaux foliaire et de la plante entière avec celles associées aux changements biochimiques et moléculaires sur la performance photosynthétique, la transpiration et l'efficacité d'utilisation de l'eau chez deux variétés (hivernales et printanières) de blé et de seigle durant l'acclimatation au froid. Chez le blé, *Arabidopsis* et *Brassica napus* l'expression des gènes W*cor* et l'acquisition de la tolérance aux basses températures sont régulés par les facteurs de transcription CBF/DREB1 et CAMTA. Au niveau biochimique et physiologique, l'acclimatation au froid des espèces herbacées tolérantes aboutit à une hausse des taux de saturation lumineuse de la photosynthèse (Asat) qui est corrélée avec l'activité des enzymes clés telles que la Rubisco, la fructose 1,6 biphosphatase et la sucrose phosphate synthase lorsque mesurées sur la base de la surface foliaire. Le phénotype court, caractéristique des plantes tolérantes acclimatés au froid semble être contrôlé par la sur-expression de CBF3 chez *Arabidopsis thaliana* et par la surexpression de *BNCBF17* chez *Brassica napus* alors que la hausse de l'épaisseur est attribuable à l'augmentation de la taille des cellules du mésophylle et /ou de celle du nombre de couches de palissade. Au niveau structurel, l'acclimatation au froid chez le seigle d'hiver et *Arabidopsis thaliana* entraîne une hausse du volume cytoplasmique et une diminution du volume vacuolaire ainsi qu'une hausse du contenu en sucrose et d'autres hydrates de carbone structurels. L'intégration des changements

morphologiques observés aux niveaux de la feuille et de la plante entière avec ceux observés aux niveaux biochimique et moléculaire durant l'acclimatation au froid sera très utile dans la compréhension des effets des concentrations élevées de CO_2 sur la croissance et le développement des plantes en fonction de la température.

Le second objectif a été de déterminer chez les cultivars de printemps et d'hiver si l'acclimatation au froid peut maintenir la stimulation à court terme de la photosynthèse par les concentrations élevées de CO_2. Les variétés de printemps lorsque acclimatées au froid sont sujettes à la photoinhibition photosynthétique contrairement aux variétés d'hiver tolérantes au froid qui arrivent à maintenir leur taux de photosynthèse. Par ailleurs, à court terme les plantes terrestres simples de type C3 perçoivent et répondent directement à l'augmentation de la concentration de CO_2 par une augmentation du taux de carboxylation de l'enzyme Ribulose 1,5 biphosphate carboxylase/oxygénase, par une diminution de l'ouverture, de la conductance et de la transpiration des stomates. Cette réponse directe aux concentrations élevées de CO_2 entraîne une amélioration de l'efficacité de l'utilisation de l'eau créant ainsi des rendements photosynthétiques plus élevés et une meilleure efficacité d'utilisation de la lumière.

Dans le troisième article de ce travail réalisé uniquement chez le blé d'hiver, le premier objectif a été de déterminer si l'acclimatation au froid peut maintenir la stimulation à long terme de la photosynthèse par les concentrations élevées de CO_2. L'exposition à long terme des plantes C3 aux concentrations élevées de CO_2 peut résulter en une acclimatation physiologique impliquant une diminution dans leur capacité d'assimilation du CO_2. Par la suite l'étude va consister à l'identification et la comparaison des gènes régulés par le CO_2 chez le blé non acclimaté et acclimaté au froid. L'exposition à un stress préalable peut conférer ou diminuer la résistance face à un autre stress. Est-ce que l'acclimatation au froid peut conférer un avantage pour la

croissance du blé en concentrations élevées de CO_2. Ces études nous permettront d'identifier la nature des gènes régulés par le CO_2 chez le blé et de les classer en fonction de leurs catégories. Cette classification donnera une image des voies métaboliques affectées par les concentrations élevées de CO_2 afin de prévoir des stratégies à adopter pour améliorer la productivité avec les changements climatiques.

CHAPITRE II

ARTICLE I: THE EFFECTS OF PHENOTYPIC PLASTICITY ON PHOTOSYNTHETIC PERFORMANCE IN WINTER RYE, WINTER WHEAT AND BRASSICA NAPUS

Keshav Dahal, Khalil Kane, Winona Gadapati, Elizabeth Webb, Leonid V Savitch, Jasbir Singh, Pooja Sharma, Fathey Sarhan, Fred J Longstaffe, Bernard Grodzinski, Norman PA Hüner.

Article publié dans le journal *Physiologia plantarum. 2012. Volume 11, issue 2. Pages 169-188*

Contribution

J'ai participé dans quelques étapes d'expérimentation et de rédaction de l'article sous la supervision et le soutien de mon directeur Fathey Sarhan et de mes collaborateurs, Keshav Dahal et des professeurs Norman Hüner et Bernard Grodzinski. J'ai effectué les expériences de PCR en temps réel et de western blot des protéines CORs régulés par le froid. J'ai été impliqué dans la correction du manuscrit avant la soumission.

2.1 RÉSUMÉ

Nous avons comparé la contribution des effets de la plasticité phénotypique observé au niveau des feuilles et de la plante entière avec les effets biochimiques et moléculaires sur la performance photosynthétique et l'utilisation de l'eau des cultivars hivernales et printanières de blé et de seigle non-acclimaté (20^0C) et acclimaté au froid (5^0C). Le blé et le seigle d'hiver (cv Norstar et Musketer) acclimaté au froid présentent des hausses de 22 et 44% de leur activité photosynthétique maximale (Asat) et du double de leur efficacité d'utilisation de l'eau. Les sucres produits de la photosynthèse sont exportés au niveau des organes de réserve et de la paroi cellulaire. Par ailleurs, les analyses biochimiques et moléculaires montrent que les céréales d'hiver acclimatées au froid augmentent leur efficacité et taux de transfert d'électrons photosynthétiques, diminuent la pression d'excitation au niveau de leur photosystème II et augmentent la dissipation de l'énergie sous forme de chaleur, démontrant ainsi une meilleure performance face à l'inhibition photosynthétique associé au froid. Par ailleurs les résultats obtenus durant la surexpression de *BNCBF17* chez *Brassica napus* indiquent que les gènes CBFs/DREBs semblent réguler la tolérance au gel et gouvernent l'architecture des plantes, l'anatomie des feuilles, la performance photosynthétique et l'efficacité de l'utilisation de l'eau. Nous discutons dans cet article des coûts et des bénéfices de la plasticité phénotypique en termes de survie hivernale et de la capacité de reproduction.

2.2 ABSTRACT

The contributions of phenotypic plasticity to photosynthetic performance in winter (cv Musketeer, cv Norstar) and spring (cv SR4A, cv Katepwa) rye (*Secale cereale*) and wheat (*Triticum aestivum*) cultivars grown at either 20°C (non-acclimated) or 5°C (cold acclimated) were assessed. The 22-40% increase in light-saturated rates of CO_2 assimilation in cold acclimated versus nonacclimated winter cereals were accounted for by phenotypic plasticity as indicated by the dwarf phenotype and increased specific leaf weight. However, phenotypic plasticity could not account for: (1) the differential temperature sensitivity of CO_2 assimilation and photosynthetic electron transport; (2) the increased efficiency and light-saturated rates of photosynthetic electron transport or (3) the decreased light sensitivity of excitation pressure and non-photochemical quenching between cold acclimated and non-acclimated winter cultivars. Cold acclimation decreased photosynthetic performance of spring relative to winter cultivars. However, the differences in photosynthetic performances between cold acclimated winter and spring cultivars were dependent upon the basis on which photosynthetic performance was expressed. Overexpression of *BNCBF17* in *Brassica napus* generally decreased the low temperature sensivity (Q_{10}) of CO_2 assimilation and photosynthetic electron transport even though the latter had not been exposed to low temperature. Photosynthetic performance in WT compared to the *BNCBF17*- overexpressing transgenic *Brassica napus* indicated that *CBFs/DREBs* regulate not only freezing tolerance but also govern plant architecture, leaf anatomy, and photosynthetic performance. The apparent positive and negative effects of cold acclimation on photosynthetic performance are discussed in terms of the apparent costs and benefits of phenotypic plasticity, winter survival and reproductive fitness.

2.3 INTRODUCTION

At the molecular level, cold acclimation induces the expression of cold-regulated (COR) genes and subsequent accumulation of corresponding proteins associated with freezing tolerance. Cold-induced genes have been identified in many plant species such as *Arabidopsis thaliana,* barley, wheat and *Brassica napus* (Sarhan *et al.,* 1997, Thomashow 2001, Chinnuswamy *et al.,* 2007, Rapacz *et al.,* 2008). In wheat and other cereals, cold acclimation-induced expression of several genes is positively correlated with the capacity of each genotype to develop freezing tolerance. Among these, the *Wcs* and *Wcor* gene families are coordinately regulated by low temperature at the transcriptional level and winter cereals exhibit higher levels of expression than do spring cereals (Sarhan *et al.,* 1997). Previous studies in *Arabidopsis thaliana*, *Brassica napus* and wheat revealed a family of CBF/DREB1 transcriptional activators (Jaglo-Ottosen *et al.,* 1998, Thomashow 2001, Savitch *et al.,* 2005, Badawi *et al.,* 2008) and CAMTA transcription factors (Doherty *et al.,* 2009) that are crucial in cold-regulation of gene expression and subsequent freezing tolerance. However, Gray *et al.,* (1997) showed that the attainment of maximum freezing tolerance in winter rye is the result of a complex interaction between light and low temperature acclimation (Gray *et al.,* 1997). In fact, the expression of *Wcs19*, previously assumed to be regulated by low temperature, is regulated by excitation pressure (Gray *et al.,* 1997).

At the biochemical and physiological levels, the effects of cold acclimation on the structure and function of the photosynthetic apparatus has been extensively studied in cold-tolerant winter cultivars of wheat, rye and barley as well as spinach, *Arabidopsis thaliana*, and *Brassica napus* (Krause 1988, Hüner *et al.,* 1993, Adams *et al.,* 2002, Stitt and Hurry 2002, Öquist and Hüner 2003, Ensminger *et al.,* 2006). Cold acclimation requires long-term growth and development at low temperature (5°C) in contrast to cold stress which can be imposed within minutes by a rapid,

short-term shift from a moderate temperature (20° - 25°C) to a low temperature regime. Based on these distinctions, cold acclimation of cold tolerant plant species generally results in enhanced light-saturated rates of photosynthesis (A_{sat}) whereas cold stress inhibits A_{sat} compared to NA controls when measured on a leaf area basis (Hurry and Hüner 1991, Boese and Hüner 1992, Öquist *et al.,* 1993, Hurry *et al.,* 1994, 1995, Hüner *et al.,* 1998, Savitch *et al.,* 2002). The cold acclimation-induced increase in A_{sat} of cold-tolerant herbaceous species is correlated with a stimulation of carbon metabolism as a result of the enhanced activities of key regulatory photosynthetic enzymes such as Rubisco, cFBPase, and SPS in response to low growth temperatures when measured on a leaf area basis (Hurry *et al.,* 2000, Stitt and Hurry 2002). There is a strong, positive correlation between the cold acclimation-induced increase in A_{sat} and the development of freezing tolerance as well as an increased resistance to low temperature-induced photoinhibition in spinach (Krause 1988, Somersalo and Krause 1989, Boese and Hüner 1992), winter rye and winter wheat (Hüner *et al.,* 1993, Öquist *et al.,* 1993, Pocock *et al.,* 2001) and *Arabidopsis thaliana* (Savitch *et al.,* 2001). Although spring cereals can grow and develop at low temperatures, unlike winter cultivars, cold acclimation decreases A_{sat} and spring cultivars remain susceptible to low temperature-induced photoinhibition of photosynthesis (Hüner *et al.,* 1993, Hurry *et al.,* 1996).

At the leaf and whole plant levels, cold acclimation of winter rye (Hüner *et al.,* 1981, 1985), spinach (Boese and Hüner 1990) as well as *Arabidopsis thaliana* (Strand *et al.,* 1999, Gorsuch *et al.,* 2010a, 2010b) results in significant alterations in plant phenotype. Growth and development of cold-tolerant plants at low temperature generally results in a compact, dwarf growth habit with leaves that exhibit increased thickness relative to NA controls. The dwarf growth habit appears to be controlled by overexpression of *CBF3* in *Arabidopsis thaliana* (Gilmour *et al.,* 2000a, 2000b) and overexpression of *BNCBF17* in *Brassica napus* (Savitch *et al.,* 2005). The increased

leaf thickness associated with the cold acclimated state can be accounted for by either increases in leaf mesophyll cell size (Hüner *et al.,* 1981, Gorsuch *et al.,* 2010a) and/or increases in the number of palisade layers (Boese and Hüner 1990). At the ultrastructural level, cold acclimated winter rye and *Arabidopsis thaliana* exhibit an apparent increase in cytoplasmic volume and an apparent decrease in vacuolar volume (Hüner *et al.,* 1984, Strand *et al.,* 1999). This is accompanied by an increase in the content of sucrose and other structural carbohydrates (Steponkus 1984, Guy 1990, Guy *et al.,* 1992, Hurry *et al.,* 1995, Strand *et al.,* 1997, 2003, Savitch *et al.,* 2000, Gorsuch *et al.,* 2010b).

Research in plant cold acclimation has focussed primarily on events at the molecular and biochemical levels (Sarhan *et al.,* 1997, Hüner *et al.,* 1998, Thomashow 2001, Stitt and Hurry 2002, van Buskirk and Thomashow 2006, Wilson *et al.,* 2006, Chinnuswamy *et al.,* 2007, Rapacz *et al.,* 2008). Although the regulation of photosynthesis, respiration and transpiration in a changing environment are critical for plant survival and productivity, few studies have attempted to integrate changes observed at the leaf and whole plant levels with those at the biochemical and molecular levels during cold acclimation (Lapointe and Hüner 1993, Gilmour *et al.,* 2000a, Savitch *et al.,* 2005, Gorsuch *et al.,* 2010a, 2010b). The objective of this study was to delineate the potential contributions of phenotypic plasticity at the leaf and whole plant levels versus those at the biochemical and molecular levels to the photosynthetic performance, transpiration and water use efficiency of winter and spring cultivars of rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) during cold acclimation. To accomplish this objective, we measured leaf morphology (stomatal density, leaf thickness), transpiration, and light- / CO_2-dependence of photosynthetic gas exchange using three frames of reference (leaf area, leaf Chl, leaf dry weight). These measurements were coupled with photosynthetic gene expression, polypeptide accumulation and in vivo Chl a fluorescence quenching analyses in NA

and CA winter and spring cultivars of wheat and rye as well as WT and a *BNCBF17*-overexpressing line of *Brassica napus*. The apparent energetic costs and benefits of phenotypic plasticity are discussed in terms of the potential for winter survival and subsequent reproductive fitness.

2.4 RESULTS

2.4.1 Effects of cold acclimation on phenotypic plasticity of rye and wheat cultivars

Except for Musketeer winter rye, minimal data are available for the growth and photosynthetic characteristics of Norstar winter wheat, SR4A spring rye and Katepwa spring wheat grown at either 20°C or 5°C. Therefore, comparative growth kinetics were performed to ensure that all comparisons of photosynthetic measurements, biochemical and molecular analyses for NA and CA plants were assessed at comparable physiological stage of development. The exponential growth rates for all CA cultivars were about 1/3 of those observed for NA counterparts (Table 1). The third leaves of all NA cultivars were fully expanded between 20-25 days and those of CA cultivars between 70-80 days. Consequently, all subsequent comparisons were made in fully expanded third leaves of 25-day-old NA and 75-day-old CA plants for all cultivars. With respect to plant morphology, CA Musketeer winter rye and Norstar winter wheat exhibited a dwarf, compact growth habit whereas NA counterparts exhibited the typical elongated growth habit (see Appendix S2 in Supporting Information). In contrast, growth of SR4A spring rye and Katepwa spring wheat resulted in an elongated growth habit irrespective of growth temperature (see Appendix S2 in Supporting Information).

Cold acclimation significantly reduced the leaf water content of the winter cultivars, Musketeer rye and Norstar wheat, by about 20% and, as a result, the dry weight to fresh weight ratio (Dwt : Fwt) increased by about 150% in both cultivars (Table 1). In contrast, cold acclimation had minimal effects on leaf water content, and thus Dwt: Fwt ratio, in the spring cultivars, SR4A rye and Katepwa wheat (Table 1). Consequently, the specific leaf weight (SLW, g dry weight m^{-2} leaf area) increased by

about 2.5-fold in CA versus NA winter cultivars whereas the SLW changed minimally between CA and NA spring cultivars (Fig. 1). Cold acclimation resulted in a 23% increase in the shoot/root ratio in CA compared to NA winter rye, but no significant changes in this ratio were observed upon cold acclimation of the other cultivars tested (Table 1). CA Norstar and Musketeer exhibited a 1.21-1.44-fold increase in Chl per leaf area relative to NA counterparts with no changes in Chl a/b. Minimal changes in Chl per unit area were observed upon cold acclimation of the spring cultivars tested (Table 1).

We observed a comparable leaf protein content of 2.3 - 3.3 g m^{-2} leaf area in all cultivars tested when grown under NA conditions (Table 1). However, CA winter wheat and winter rye exhibited a 4.2 to 4.7–fold increase in total leaf protein content when measured on a leaf area basis and a 3.3 to 3.5-fold increase in the protein/Chl ratio compared to NA controls (Table 1). In contrast, CA spring wheat and spring rye exhibited minimal changes in total leaf protein content per leaf area and protein/Chl ratio relative to NA controls (Table 1). The effects of growth temperature on total plant dry matter accumulation and plant morphology were not affected by variations in pot size (see Appendix S3 in Supporting Information).

2.4.2 Effects of cold acclimation on CO2 assimilation in rye and wheat cultivars

Since leaf surfaces regulate photosynthetic CO_2 gas exchange, rates of photosynthetic CO_2 assimilation are typically calculated on a per unit leaf area basis. All cultivars exhibited comparable light-saturated rates of net CO_2 assimilation (A_{sat}) of 17- 20 μmol CO_2 m^{-2} s^{-1} when grown and measured at 20°C and measured on a leaf area basis (Table 2, A_{sat}, NA, 20). Cold stress imposed by an immediate reduction in the measuring temperature from 20° to 5°C inhibited these rates by about 35 - 50% in all NA winter and spring cereals (Table 2, A_{sat}, NA, 5). However, the CA winter cultivars, Musketeer rye and Norstar wheat, were able to maintain net CO_2

assimilation rates at 5°C (Table 2, A_{sat}, CA, 5) that were comparable to or slightly higher than those rates observed for NA controls but measured at 20°C (Table 2, A_{sat}, NA, 20). Light- saturated rates of photosynthesis of CA winter cultivars measured at 20°C (Table 2, A_{sat}, CA, 20) were 16-20% higher than those measured at 5°C (Table 2, A_{sat}, CA, 5). In addition, rates of net CO_2 assimilation measured at 5°C (Table 2, A_{sat}, CA, 5) resulted in 60 - 80% higher rates in CA Musketeer and Norstar compared to NA controls measured at 5°C (Table 2, A_{sat}, NA, 5). Thus, cold acclimation of Musketeer and Norstar enhanced light-saturated rates of net CO_2 assimilation on a leaf area basis irrespective of the measuring temperature.

In contrast to the CA winter cultivars, the CA spring cultivars, SR4A rye and Katepwa wheat, exhibited a 30 - 35% decrease in the light-saturated rates of net CO_2 assimilation (Table 2, A_{sat}, CA, 5) relative to NA controls (Table 2, A_{sat}, NA, 20) when measured on a leaf area basis. The assimilation rates observed for CA spring cultivars were comparable to those rates observed for NA controls that were cold-stressed at 5°C. Hence, the CA-induced increase in light-saturated rates of net CO_2 assimilation measured on a leaf area basis appeared to be specific for the winter cultivars tested.

The data in Fig. 2 illustrate the temperature dependence of light-saturated rates of net CO_2 assimilation in winter Musketeer and spring (SR4A) rye. Although both NA and CA Musketeer exhibited a temperature maximum of about 25°C, the low temperature sensitivity of CO_2 assimilation rates (5° to 25°C) was less for CA (Q_{10} = 1.05) than that of NA Musketeer (Q_{10} = 1.38) (Fig. 2A). However, CO_2 assimilation in CA Musketeer exhibited minimal differences in sensitivity to high temperature (25° to 40°C) from that of NA Musketeer (Fig.2A). In contrast to winter rye, CA spring rye exhibited lower rates of CO_2 assimilation at all temperatures between 15° and 40°C than that of NA spring rye (Fig. 2B). Thus, although NA Musketeer and

SR4A exhibited similar temperature profiles for net CO_2 assimilation, CA winter rye maintained rates of photosynthesis that were about 1.8-fold higher than NA winter rye as well as CA SR4A at temperatures between 5° and 25°C when measured on a leaf area basis (Fig. 2A). These results are consistent with the data in Table 2, which indicated that cold acclimation of SR4A caused an inhibition of CO_2 assimilation whereas CA Musketeer exhibited a stimulation of CO_2 assimilation relative to NA counterparts.

When CO_2 assimilation rates were calculated on a per unit Chl basis (Fig. 2C), CA Musketeer exhibited higher rates at temperatures below 15°C whereas NA plants exhibited higher rates of photosynthesis at temperatures above 15°C. Similar trends were observed for Norstar winter wheat (see Appendix S4 in Supporting Information). In contrast to Musketeer, calculation of CO_2 assimilation rates on a per unit Chl basis had minimal effects on the temperature response profiles for SR4A spring rye (compare Fig. 2B and 2D) as well as Katepwa spring wheat (see Appendix S4 in Supporting Information).

The difference in the calculated Q_{10} for CO_2 assimilation between 5° and 25°C for CA and NA Musketeer was unaffected by the method of calculating light-saturated rates of photosynthesis. However, when light-saturated rates of CO_2 assimilation in Musketeer (Fig. 2E) and SR4A (Fig. 2F) were normalized on a leaf dry weight basis, minimal differences were observed in the temperature profiles of Musketeer versus SR4A in either the cold acclimated or non-acclimated state. Similar trends were observed for Norstar winter wheat versus Katepwa spring wheat (see Appendix S4 in Supporting Data).

The results for the effects of cold acclimation on light-saturated rates of photosynthetic electron transport associated with CO_2 assimilation (Table 3, J_{max}) and

ETR (Table 3; see Appendix S5 in Supporting Data) are consistent with the results for light-saturated rates of net CO_2 assimilation based on leaf area (Table 2). CA Musketeer and Norstar generally exhibited higher J_{max} relative to NA controls irrespective of the measuring temperature. However, J_{max} was inhibited by 14-20% upon exposure to a low measuring temperature in NA controls whereas it was inhibited by only 3-8% in CA winter cultivars (Table 3, 20 vs 5). Consequently, the Q_{10} for J_{max} in CA Musketeer (1.06) and Norstar (1.01) were lower than those of the NA rye (1.16) and wheat counterparts (1.11). Similar trends were observed when light-saturated rates of photosynthetic electron transport were calculated from Chl fluorescence data (Table 3, ETR; see Appendix S5 in Supporting Data), which are indicative of maximum rates of electron transport. Furthermore, similar trends with respect to the relative effects of cold acclimation and sensitivity to measuring temperature were observed for both apparent maximum quantum efficiency (Table 3, Q) as well as carboxylation efficiency (Table 3, CE).

In contrast to the winter cultivars, the CA spring cultivars, SR4A rye and Katepwa wheat, exhibited a significant inhibition of J_{max} and ETR relative to NA controls (Table 3; see Appendix S5 in Supporting Data). These results are consistent with the inhibition of light-saturated rates of CO_2 assimilation induced by cold acclimation of SR4A spring rye and Katepwa spring wheat (Table 2). J_{max} and ETR observed for CA spring cultivars were comparable to those rates observed for NA controls measured at 5°C. However, both CA winter and spring cultivars exhibited decreased sensitivity of CO_2 assimilation (Fig. 2; see Appendix S4 in Supporting Data) as well as ETR (see Appendix S5 in Supporting Data) to low measuring temperatures between 5° and 25°C relative to NA control plants.

2.4.3 Differential effects of increased SLW and Chl on photosynthetic light and CO2 response curves.

Increases in light-saturated rates of photosynthetic CO_2 assimilation between NA and CA winter rye and wheat based on leaf area ignores potential contributions of differences in SLW, Chl per unit area and leaf thickness to CO_2 gas exchange. Since cold acclimation caused a 2.5-fold and 1.44-fold increase in SLW and Chl per unit area in winter Musketeer rye (Fig. 1, Table 1), we tested the possible contributions of increased SLW and Chl per unit leaf area on the light (Fig. 3) and CO_2 response curves (Fig. 4) for NA and CA rye cultivars when measured at the same temperature (20°C). The results illustrated in Fig. 3 indicate that, although light-saturated rates of CO_2 assimilation were about 1.4-fold higher in CA than in NA Musketeer when expressed on the leaf area basis (Fig. 3A), this difference was eliminated when CO_2 assimilation was expressed on a Chl basis (Fig. 3C). Furthermore, the light-saturated rates of CO_2 assimilation were approximately 50% lower in CA than in NA Musketeer when expressed on a leaf dry weight basis (Fig. 3E). Since the growth of spring rye, SR4A, at low temperature resulted in minimal changes on Chl per unit area (Table 1), cold acclimation of spring rye, SR4A, inhibited light-saturated rates of CO_2 assimilation irrespective of whether CO_2 assimilation was expressed either on a leaf area (Fig. 3B) or per unit Chl basis (Fig. 3D). However, the light response curves for CA and NA Musketeer were indistinguishable from those of CA and NA SR4A when calculated on a leaf dry weight basis (Fig. 3E and 3F). Similar trends were observed for the CO_2 response curves for NA and CA Musketeer winter rye (Fig. 4A, C and E) and NA and CA spring rye (Fig. 4B, D and F). Comparable results were observed for winter and spring cultivars of wheat (see Appendix S6 and S7 in Supporting Information).

2.4.4 Effects of cold acclimation on stomatal conductance and water use efficiency

Since the rates of leaf gas exchange and rates of water loss through transpiration are under stomatal control, we examined the effects of cold acclimation on stomatal conductance (gs) and stomatal density. Cold acclimation suppressed the stomatal conductance by 60 - 70% in the winter cultivars, Musketeer and Norstar, when measured at 20°C but by 30-35% when measured at 5°C (Table 4). As a result, leaf transpiration rates were 35 - 50% lower in these cultivars during cold acclimation irrespective of the measuring temperature (Table 4). Thus, the stimulation of light-saturated (Fig. 3A) and CO_2-saturated rates of CO_2 assimilation in CA winter cultivars (Fig. 4A) measured on a leaf area basis can not be due to a stomatal effect.

In contrast to the winter cultivars, cold acclimation suppressed the stomatal conductance by 30 - 40% and transpiration rates by about 20% in the spring cultivars, SR4A and Katepwa (Table 4). Furthermore, stomatal conductance of the CA winter cultivars appeared to be less temperature sensitive than their NA counterparts whereas CA and NA spring cultivars appeared to be equally sensitive to measuring temperature. Consequently, CA Norstar and Musketeer exhibited approximately a 3-fold increase in leaf water use efficiency (WUE) relative to NA controls whereas CA Katepwa and SR4A exhibited a minimal change in leaf WUE relative to NA controls irrespective of the measuring temperature (Table 4).

It appears that the suppressed stomatal conductance observed in CA plants can be accounted for, in part, by a decrease in stomatal density on both the abaxial and adaxial leaf surfaces for all four cultivars tested. For example, CA Musketeer winter rye exhibited a 55% reduction in adaxial stomatal density (72 ± 22 stomates mm^{-2}) compared to NA Musketeer (159 ± 14 stomates mm^{-2}). In contrast, CA SR4A spring rye exhibited only a 27% reduction in adaxial stomatal density (101 ± 15) compared to NA SR4A (137 ± 6). Similar trends were observed for the effects of cold

acclimation on adaxial and abaxial stomatal densities in Norstar winter wheat compared to Katepwa spring wheat (data not shown). These data indicate that the differential enhancement of WUE induced by cold acclimation of Musketeer winter rye and Norstar winter wheat is associated with a decrease in stomatal density in addition to increased A_{sat}.

2.4.5 Effects of cold acclimation on δ13C values of leaf tissues

Alterations in stomatal density and WUE should be reflected in changes in $^{13}C/^{12}C$ carbon isotope ratios measured as $\delta^{13}C$ (‰) (O'Leary 1988). The results illustrated in Table 1 indicate that CA Musketeer exhibited a 2.3‰ increase (less negative) in $\delta^{13}C$ compared to NA winter rye. In contrast, we detected no significant differences in the $\delta^{13}C$ values between CA and NA SR4A (Table 1). These results are consistent with the differential effects of cold acclimation on WUE in winter versus spring cultivars (Table 4).

2.4.6 Effects of cold acclimation on dark respiration rates

Respiration is another important process that contributes to leaf net CO_2 gas exchange. All four cultivars exhibited comparable dark respiration rates of about 2.1 - 3.2 μmol CO_2 evolved $m^{-2}s^{-1}$ at 20°C and ambient CO_2 (Table 2, R_{dark}, NA, 20). An immediate reduction in measuring temperature from 20° to 5°C inhibited these rates by 15 – 25% in all NA cultivars (Table 2, R_{dark}, NA, 5). However, unlike photosynthesis, cold acclimation significantly increased dark respiration rates (17 – 30%) not only in winter cultivars but also in spring cultivars irrespective of the measuring temperatures (Table 2, R_{dark}, CA). Thus, we did not detect any differential

effects of cold acclimation on rates of respiration in winter versus spring cultivars of rye and wheat.

2.4.7 Effects of cold acclimation on ETR, EP and NPQ

In vivo Chl a fluorescence provides a convenient means to quantify maximum potential rates of photosynthetic electron transport (ETR), excitation pressure (EP), which estimates the relative reduction state of PSII reaction centers, and non-photochemical quenching (NPQ), an estimate of the capacity to dissipate energy as heat. However, differences in leaf morphology have minimal impact on measurements of ETR, EP and NPQ by Chl a fluorescence. The amount of light absorbed by Chl decreases as the light penetrates into the leaf with the fluorescence signal being strongest near the leaf surface. Light response curves for 1-qP and NPQ are useful to estimate the apparent quantum requirement to close PSII reaction centers and to induce energy dissipation by NPQ, respectively (Rosso *et al.,* 2009). Apparent quantum requirement is the inverse of apparent quantum efficiency and is estimated as the inverse of the initial slopes calculated from the linear portion of the light response curve for either 1-qP (Fig. 5 C and D) or NPQ (Fig. 5 E and F). Fig. 5 illustrates the light response curves of ETR, 1-qP and NPQ for NA and CA Musketeer winter and SR4A spring rye. The light response curves for ETR in Musketeer indicate that cold acclimation stimulated both the apparent quantum efficiency (maximum initial slope) as well as the light-saturated rates of photosynthetic electron transport by about 50% relative to NA Musketeer (Fig. 5A). This was associated with a 13% increase in the apparent quantum requirement for PSII closure (Fig. 5C) coupled with a concomitant 40% increase in the apparent quantum requirement to induce NPQ (Fig. 5E) in CA versus NA Musketeer. In contrast, cold acclimation of spring rye, SR4A, resulted in a 25% decrease in the apparent quantum efficiency as well as the light- saturated rates of ETR (Fig. 5B). This was associated with a 40% decrease in the apparent quantum requirement for

PSII closure (Fig. 5D) and a 12% decrease in the apparent quantum requirement to induce NPQ (Fig. 5F) in CA relative to NA SR4A. Similar trends in ETR, EP and NPQ were observed in NA and CA Norstar winter wheat versus NA and CA Katepwa spring wheat (see Appendix S8 in Supporting Information).

2.4.8 Effects of cold acclimation on photosynthetic gene expression and polypeptide accumulation

To confirm that the winter and spring cultivars tested were differentially cold acclimated, the accumulation of three COR protein markers (Wcs120, Wcor410 and Wcs19) in Norstar winter and Katepwa spring wheat were measured. None of the COR proteins were detected in either wheat cultivars in the NA state (see Appendix S9, lane 1 and 3 in Supporting Information). However, we observed the accumulation of all three COR proteins tested in CA Norstar but not in CA Katepwa (see Appendix S9, lane 2 and 4 in Supporting Information). Therefore, the differential accumulation of COR proteins in winter versus spring wheat cultivars in response to low growth temperature confirms that these cereals were indeed differentially cold acclimated.

Cold acclimation also appeared to alter photosynthetic gene expression. Transcript abundance either increased (*rbcL*, *cFBPase*, *psbA*) or remained about the same (*Lhcb1*, *psaA*) in CA versus NA Norstar wheat (Fig. 6A). Similar trends were observed in CA Katepwa spring wheat except that *cFBPase* expression was significantly inhibited during cold acclimation (Fig. 6A). However, at the protein level, cold acclimation induced minimal changes (10 - 15%) in the relative amount of major photosynthetic enzymes and components of photosynthetic electron transport such as rbcL, cFBPase, Lhcb1, psbA and psaA in either Musketeer winter rye (Fig. 6B, Musketeer, CA versus NA) or Norstar winter wheat (Fig. 6B, Norstar, CA versus NA) when expressed on total leaf protein basis. However, cold acclimation appeared to reduce the level of psbA, the PSII reaction center polypeptide, in both SR4A (59%)

(Fig. 6B, SR4A, CA versus NA) and Katepwa (32%) (Fig. 6B, Katepwa, CA versus NA). Minimal changes in both *Lhcb1* expression and Lhcb1 accumulation are consistent with minimal differences in the Chl a/b ratios between CA and NA winter and spring cultivars (Table 1). We conclude that the levels of rbcL and cFBPase as well as the other components of the photosynthetic apparatus are 3-fold greater in CA than NA winter cereals on the basis of a 3-fold increase in the protein/Chl ratio (Table 1). In contrast, this was not observed in the spring varieties since cold acclimation affected the protein/Chl ratio minimally in these cultivars (Table 1).

2.4.9 Effects of over-expression of BNCBF17 on phenotypic plasticity and photosynthetic performance in Brassica napus

Overexpression of *CBFs* induces a dwarf growth habit and increases freezing tolerance in *Arabidopsis thaliana* (Gilmour *et al.*, 2000a, 2000b) as well as *Brassica napus* (Savitch et al 2005). Fig. 7 illustrates that over-expression of *BNCBF17* in *Brassica napus* resulted in a dwarf growth habit and approximately a 1.7-fold increase in leaf thickness (330μm) compared to WT (191μm) which appeared to be primarily due to an increased thickness of the palisade layer.

The changes in leaf anatomy and plant phenotype in the *BNCBF17*-overexpressing line were associated with a 1.7-fold increase in SLW, a 2.7-fold increase in Chl per unit leaf area (Table 5) and a 1.3-fold increase in light-saturated rates of gross CO_2 assimilation measured on a leaf area basis compared to WT (Fig. 8A). This was coupled with a significant increase in apparent quantum yield (Q) of CO_2 assimilation but no significant change in carboxylation efficiency (CE) (Table 5). However, the light response curves illustrated in Fig. 8A indicate that the 1.3-fold increase in light-saturated rates of CO_2 assimilation exhibited by the *BNCBF17*-overexpressing transgenic line relative to the WT when expressed on a leaf area basis was eliminated when calculated either on a per unit Chl basis (Fig. 8C) or on a leaf

dry weight basis (Fig. 8E). Although the *BNCBF17* transgenic line exhibited a significant increase in the light and CO_2-saturated rates of CO_2 assimilation on a leaf area basis (Fig. 8B), both the carboxylation efficiency as well as the light and CO_2-saturated rates of CO_2 assimilation were lower in the *BNCBF17* transgenic line compared to WT when calculated either on a per unit Chl basis (Fig. 8D) or on a leaf dry weight basis (Fig. 8F).

Fig. 9 illustrates the temperature response curves for CO_2 assimilation (Fig. 9 A, B, C) and ETR (Fig. 9D) for WT and the transgenic *BNCBF17* line of *Brassica napus*. The *BNCBF17* transgenic line exhibited higher light-saturated rates of CO_2 assimilation (Fig. 9A) and ETR (Fig. 9D) than WT at all temperatures between 5° and 25°C when they were expressed on a leaf area basis. This difference between the WT and the *BNCBF17* transgenic line with respect to CO_2 assimilation was eliminated when CO_2 assimilation was expressed on either a Chl basis (Fig. 9B) or of a leaf dry weight basis (Fig. 9C). However, overexpression of *BNCBF17* decreased the low temperature sensitivity of CO_2 assimilation and ETR ($Q10_{Asat}$ = 1.13; $Q10_{ETR}$ = 1.07) compared to WT ($Q10_{Asat}$ = 1.47; $Q10_{ETR}$ = 1.32) (Fig. 9).

The transgenic *BNCBF17* line exhibited minimal changes in transpiration rates compared to WT (Table 5) which were consistent with minimal changes in adaxial (WT : *BNCBF17* = 0.79) and abaxial (WT : *BNCBF17* = 1.09) stomatal frequencies calculated from the SEM micrographs of respective leaf surfaces (Fig. 7, Table 5). However, WUE was 30% higher and the $\delta^{13}C$ value increased by 1.5‰ in the *BNCBF17* line compared to the WT (Table 5).

In vivo Chl a fluorescence indicated that the transgenic line exhibited a small (10%) but significant increase in light-saturated ETR but no change in the apparent quantum efficiency of ETR compared to WT (Fig. 10A). However, the apparent

quantum requirements for closure of PSII reaction centers (Fig. 10B) and for NPQ (Fig. 10C) were 25% and 35% higher, respectively, in the *BNCBF17*- overexpressing line than the WT.

2.5 DISCUSSION

In this report, we show that the conclusions drawn from a comparison of CO_2 assimilation rates of CA versus NA winter cereals are directly dependent on how one expresses the gas exchange data, that is, are dependent on the frame of reference. All three frames of reference (leaf area; leaf Chl; leaf dry weight) used to calculate photosynthetic gas exchange rates are useful but each frame of reference provides a somewhat different perspective of the effects of cold acclimation on photosynthetic performance in winter cereals. Cold acclimation of winter cereals resulted in significant reductions in stomatal conductance (Table 4) and stomatal densities on both leaf surfaces, which are associated with a 1.5-fold increase in leaf thickness (Hüner *et al.,* 1985, Gorsuch *et al.,* 2010a). Thus, CA winter rye and wheat exhibited a significant reduction in the ratio of leaf gas exchange surface area (SA) to leaf volume (V) (SA/V) as compared to NA winter rye and wheat. Despite these significant restrictions regarding the surface area available for CO_2 gas exchange, CA Musketeer (Fig. 3A; Fig. 4A) and Norstar (see Appendix S6 and S7 in Supporting Information) exhibited higher rates of light-saturated as well as CO_2-saturated CO_2 assimilation than NA winter cultivars when expressed on a leaf area basis. This is a consequence, first, of an increased protein/Chl ratio (Table 1) which reflects a change in the amount of photosynthetic apparatus at the chloroplast level, and second, of an increased SLW (Fig. 1) which reflects an increase in the amount of photosynthetic apparatus available per unit leaf area upon growth at low temperature (compare Fig. 3A and C as well as Fig. 4A and C). The enhanced photosynthetic efficiency and capacity of CA winter cereals when CO_2 assimilation is expressed on a leaf area basis is consistent with the 3-fold higher protein/Chl ratio (Table 1) and the increased quantum requirements for the closure of PSII reaction centers and the induction of energy dissipation through NPQ (Fig. 5). This means that CA winter cereals exhibit an increased efficiency to convert absorbed photons into fixed carbon and biomass

and concomitantly minimizing energy loss through NPQ. Altering the phenotype and SLW of *Brassica napus* by overexpression of *BNCBF17* (Fig. 7, Fig. 8, Fig. 9, Fig. 10) resulted in comparable enhancement of photosynthetic performance as exhibited by CA Musketeer and Norstar. In addition, CA winter cultivars exhibited an enhanced WUE (Table 4) irrespective of the measuring temperature relative to NA controls. Thus, we conclude that the phenotypic plasticity of winter cereals associated with cold acclimation, such as the induction of a dwarf growth habit, increased leaf thickness (Hüner *et al.,* 1985, Gorsuch *et al.,* 2010a) and increased SLW, contribute directly to their enhanced photosynthetic performance when rates of CO_2 assimilation are expressed on a leaf area basis.

In contrast to the photosynthetic performance of CA winter rye (Fig. 3; Fig. 4) and winter wheat (see Appendix S6 and S7 in Supporting Information), the photosynthetic performance of spring rye and spring wheat indicated that cold acclimation resulted in an inhibition of photosynthetic efficiency and photosynthetic capacity when CO_2 assimilation rates were expressed on a leaf area basis (Fig. 3; Fig. 4). This was associated with decrease in the quantum requirement for both PSII reaction center closure and the concomitant induction of NPQ (Fig. 5). This means that, upon cold acclimation, spring cereals exhibit a decreased efficiency to convert absorbed photons into fixed carbon and biomass which is compensated by an increased efficiency for the dissipation of excess absorbed energy through NPQ. In addition, CA spring cultivars did not alter WUE (Table 4) and $\delta^{13}C$ (Table 1) relative to NA controls. Furthermore, the inhibition of photosynthesis in the CA spring cultivars was associated with minimal phenotypic plasticity with respect to plant and leaf morphology (See Appendix S2 in Supporting Information) and SLW (Fig. 1).

However, comparisons of photosynthetic performance between winter and spring cultivars result in alternative conclusions when CO_2 assimilation is calculated on a

leaf dry weight basis. First, cold acclimation of winter rye caused a 50% decrease in light-saturated (Fig. 3E) as well as light and CO_2-saturated rates of CO_2 assimilation (Fig. 4E). Similar results were observed for CA versus NA winter wheat (see Appendix S6 and S7 in Supporting Information). Thus, cold acclimation of winter rye and winter wheat induced an inhibition of photosynthetic capacity relative to NA controls when expressed on a leaf dry weight basis (Fig. 3E, Fig. 4E, see Appendix S6E and S7E in Supporting Information) rather than on a leaf area basis (Fig. 3A, Fig. 4A, see Appendix S6A and S7A in Supporting Information). Second, when CO_2 assimilation rates were expressed on a leaf dry weight basis, no differences in photosynthetic performance were observed between CA winter and spring cultivars (Fig. 3; Fig. 4; see Appendix S6 and S7 in Supporting Information). CA winter and CA spring cultivars both exhibited an inhibition of photosynthetic capacity. Thus, the assessment of photosynthetic performance on a leaf dry weight basis appears to eliminate the effects of the differential phenotypic plasticity exhibited by winter and spring cultivars upon cold acclimation. A similar inhibition of photosynthetic performance is observed when WT *Brassica napus* and the *BNCBF17* overexpressing line are compared (Fig. 8E, Fig. 8F). These results provide additional support for our thesis that integration of the changes in plant morphology and leaf anatomy induced during growth at low temperature are important in the interpretation of the comparative photosynthetic performance between winter and spring cultivars during cold acclimation.

How can one rationalize the apparent conundrum between the interpretation of the results for photosynthetic performance expressed on a leaf area basis versus leaf dry weight basis in CA winter and spring cereals? We suggest that this can be explained by an analysis of the potential costs and benefits of phenotypic plasticity associated with cold acclimation. Growth at low temperature induces maximum freezing tolerance in winter cultivars through the induction of cold tolerance-

associated genes (See Appendix S9 in Supporting Information), which maximizes the probability of survival during the harsh winter conditions. In addition, a characteristic which distinguishes overwintering cereals from spring cereals is that the former increase SLW (Fig. 1) and exhibit a dwarf, compact growth habit compared to the latter (See Appendix S2 in Supporting Information). The increase in SLW can be explained, in part, by the fact that the equivalent total plant dry matter present in a NA plant accumulates in a much smaller, dwarf CA plant (Table 1; Appendix S2 in Supporting Information). Consequently, cold acclimation of winter cereals induces an increase in the ratio of plant biomass / plant volume, that is, an increase in energy per unit volume. We suggest that this reflects a requirement of leaves of cold-acclimated winter cereals to sustain an increased leaf metabolic demand and accumulation of cryoprotectants in order to maintain the cold acclimated state while concomitantly supporting translocation of photoassimilates to critical overwintering sinks such as the crown. Thus, overwintering cereals divert metabolic energy away from vegetative growth and store the fixed carbon as an energy source for the maintenance of the cold acclimated state, which is characterized by enhanced utilization of absorbed photons and increased resistance to photoinhibition (Hurry *et al.,* 1992, Pocock *et al.,* 2001) as well as increased cryoprotection and freezing tolerance. Consequently, we suggest that a cost of increasing the biomass or energy per unit plant volume is a reduction in photosynthetic capacity when measured on a leaf dry weight basis. However, this apparent cost must be considered in the context of the potential benefit of increasing the biomass or energy per unit volume and WUE, which includes a greater probability of winter survival combined with enhanced reproductive fitness in the following spring due, in part, to the energy accumulated during the cold acclimation period in the preceding autumn.

In contrast to the winter cultivars, cold tolerance-associated genes are not induced in spring cultivars (See Appendix S9 in Supporting Information) and hence

the spring cultivars are unable to divert energy from vegetative growth to storage required for successful overwintering. Consequently, CA spring cultivars show no changes in plant growth habit (See Appendix S2 in Supporting Information), exhibit minimal changes in SLW compared to NA plants (Fig. 1) and decrease the efficiency of energy conversion into biomass. Thus, spring cultivars remain susceptible to low temperature photoinhibition (Hurry *et al.,* 1992, Pocock *et al.,* 2001). Thus, the lack of phenotypic plasticity and decreased photosynthetic performance limits the ability of spring cultivars to exploit photosynthesis at low temperatures in the fall and subsequently succumb to the freezing conditions.

What governs phenotypic plasticity, photosynthetic performance and energy storage in overwintering plants? Compared to WT *Brassica napus,* overexpression of *BNCBF17* in *Brassica napus* grown at 20°C mimicked the effects of cold acclimation in Musketeer winter rye and Norstar winter wheat with respect to increased SLW (Table 5), increased leaf thickness and increased photosynthetic performance (Fig. 8A, B). This is also associated with an increase freezing tolerance (Savitch *et al.,* 2005), which confirms previous published reports for *Arabidopsis thaliana* (Lui *et al.,* 1998, Kasuga *et al.,* 1999, Gilmour *et al.,* 2000). Thus, the overexpression of *Brassica napus* with a single transcription factor, *BNCBF17,* appears to cause the conversion of non-acclimated WT to a cold acclimated state without exposure to low temperature. CBFs/DREBs are a family of transcriptional activators (Liu *et al.,* 1998, Kasuga *et al.,* 1999, Gilmour *et al.,* 2000, van Buskirk and Thomashow 2006) required for the induction of a suite of cold-regulated genes (*COR*) such as *WSC120, WCOR410* and *WSC19* (See Appendix S9 in Supporting Information) that enhance plant freezing tolerance (Sarhan *et al.,* 1997). In addition to enhanced freezing tolerance, we confirm that overexpression of *BNCBF17* has much broader effects on leaf anatomy, CO_2 assimilation, photosynthetic electron transport and dry matter accumulation (Savitch *et al.,* 2005). As observed upon cold

acclimation of winter rye and winter wheat, overexpression of *BNCBF17* induces a dwarf phenotype with a total dry matter content that is approximately equivalent to the larger, extended phenotype observed in the WT (Table 5, Fig, 7A, B). Thus, CBFs/DREBs appear to be critical factors that govern plant phenotypic plasticity during cold acclimation from the level of gene expression and freezing tolerance to whole plant architecture, to photosynthetic electron transport and CO_2 assimilation to resistance to photoinhibition. However, the governance of phenotypic plasticity and photosynthetic performance by CBFs/DREBs in winter cultivars must also be integrated with the process of vernalization (Amasino 2004, Sung and Amasino 2005).

Light-saturated CO_2 assimilation rates and ETR appear to be surprisingly insensitive to temperature between 5° and 25°C in CA compared to NA cultivars (Fig.2, see Appendix S4, S5 in Supporting Information). This was observed in both winter and spring cultivars. Thus, cold acclimation appears to stabilize enzymes of the Calvin cycle as well as the thylakoid membrane to a greater extent in CA than in NA winter and spring cultivars. In addition, we show that overexpression of *BNCBF17* in *Brassica napus* also reduces the low temperature sensitivities of light-saturated CO_2 assimilation as well as ETR (Fig. 9) in a manner comparable to that observed for cold acclimated winter cereals even though the *BNCBF17* overexpressing line had never been exposed to low temperature. This is consistent with the thesis that CBFs/DREBs are critical factors governing photosynthetic performance during cold acclimation.

Although alterations in plant morphology, leaf anatomy and SLW in CA winter cultivars as well as *BNCBF17*- overexpressing *Brassica napus* can, by and large, account for the changes in photosynthetic performance, these changes at the leaf level cannot account for the differences either in temperature sensitivities (Q_{10}) for CO_2

assimilation (Fig. 2; Fig. 9; see Appendix S4 in Supporting Information) and ETR (Fig. 9; see Appendix S5 in Supporting Information) or the differences in the light response curves for ETR, excitation pressure and NPQ in CA versus NA plants (Fig. 5, see Appendix S8) or between WT versus the *BNCBF17*-overexpressing line (Fig. 10). This indicates that growth temperature must also influence the processes of CO_2 assimilation and photosynthetic electron transport at the biochemical level. This, in part, may be a reflection of the increased amounts of the different components of the photosynthetic apparatus in CA compared to NA winter cultivars to enhance energy utilization versus energy dissipation through NPQ. This is consistent with the 3-fold increase in the protein/Chl ratio and the increased quantum requirement for not only PSII closure but also the induction of NPQ (Fig. 5, see Appendix 8 in Supporting Information). Consequently, CA Musketeer and Norstar exhibited a lower excitation pressure for a given irradiance, and thus, a greater capacity to keep Q_A oxidized than NA plants (Fig. 5, see Appendix 8 in Supporting Information). These results are consistent with published data, which indicate an increased resistance to low temperature-induced photoinhibition in CA versus NA winter cultivars and consistent with the fact that winter and spring cultivars can be discriminated from each other on their differential sensitivities to photoinhibition (Hurry and Hüner 1992, Pocock *et al.*, 2001). Lapointe and Hüner (1993) reported that intact, photosynthetically active, mesophyll cells isolated from cold acclimated rye leaves exhibited a similar increased resistance to photoinhibition as observed for intact CA rye leaves. They concluded that the increased resistance to photoinhibition observed in cold acclimated rye must reside at the cellular level rather than at the level of the intact leaf (Lapointe and Hüner 1993). Thus, both changes in Q_{10} and the decreased efficiencies of PSII closure and the induction of NPQ as a function of irradiance in either CA versus NA rye and wheat or in WT versus the *BNCBF17*- overexpressing line reflect, in part, the inherent flexibility in energy utilization by photosynthetic electron transport (McDonald *et al.*, 2011) rather than changes in leaf anatomy and plant phenotype.

2.6 MATERIALS AND METHODS

2.6.1 Plant materials and growth conditions

One cultivar each of winter (cv Norstar) and spring (cv Katepwa) wheat (*Triticum aestivum* L.) and one each of winter (cv Musketeer) and spring (cv SR4A) rye (*Secale cereale* L) was used. Seeds were germinated and grown in the controlled environmental growth chambers (Model: GCW15 chamber, Environmental Growth Chambers, Chargin Falls, Ohio, USA) at ambient CO_2 (380 ± 15 ppm), a PPFD of 250 ± 20 µmol photons m^{-2} s^{-1}, 55 ± 5% relative humidity, a 16 h photoperiod and at day/night temperature regimes of 20/16°C for non-acclimated (NA) and 5/5°C for cold acclimated (CA) plants. The temperature, relative humidity, irradiance level and photoperiod in each chamber were computer-controlled and monitored and recorded continuously. Rye and wheat seedlings were grown in coarse vermiculite in 500mL-sized plastic pots at a density of three plants per pot and watered with Hoagland's solution.

Brassica napus cv. Westar (WT) and the *Brassica napus BNCBF17*-overexpressing transgenic line, generated as described in detail elsewhere (Savitch *et al.,* 2005), were grown in controlled environmental growth chambers (Model: GCW15 chamber, Environmental Growth Chambers, Chargin Falls, Ohio, USA) with a 16 h photoperiod and at day/night temperature regimes of 20/16°C as described above except that the seedlings were grown in organic soil (Promix) in 300mL-sized pots with one plant each and watered with all-purpose fertilizer (Plant Prod 20-20-20, Sure-Gro IP Inc. Brandford, ON, Canada). The WT plants were grown for 3 weeks and the *BNCBF17* overexpressing line for 4 weeks. At these ages, the third leaves were fully expanded in both WT and the transgenic plants.

2.6.2 Comparative growth kinetics

Since minimal data are available for the growth and photosynthetic characteristics of NA and CA Norstar winter wheat, Katepwa spring wheat and SR4A spring rye, comparative growth kinetics were performed to ensure that all comparisons of photosynthetic measurements and biochemical analyses between NA and CA plants were assessed at a comparable developmental stage. NA plants were harvested every week and CA plants every two weeks until the full stem elongation stage. Total tiller number, leaf number and leaf blade area were recorded on each harvest. Total root and shoot fresh biomass were weighed, and the tissues were dried at 80°C to constant weight for the determination of dry weight. Exponential growth rates (g dry mass day^{-1}) were calculated from plots of the natural logarithm (*ln*) of shoot dry mass over time in days. Specific leaf weight (SLW) was calculated as leaf dry weight in g m^{-2} leaf blade area. Leaf blade area was measured by using a LI-COR portable area meter (LI–3000A, LI-COR Biosciences, Lincoln, Nebraska, USA). The photosynthetic pigments, chlorophyll a and b, were determined according to Arnon (1949).

2.6.3 Scanning electron microscopy

In order to assess the effect of *BNCBF17* overexpression on *Brassica napus* leaf anatomy, leaf surface and leaf cross sections were examined by scanning electron microscope. Leaf samples from WT and the *BNCBF17*-overexpressing line were cut into small pieces ($2mm^2$) and fixed in 2.5% (w/v) glutaraldehyde: 2% (w/v) paraformaldehyde in 20 mM phosphate buffer (pH 7.2) for 12 h at 4°C. The samples were washed and postfixed in 2% (v/v) osmium tetroxide in the same phosphate buffer for 2 h at room temperature. The samples were then washed thoroughly in the buffer and dehydrated in graded series of alcohol (50%, 70%, 90% and 100%) for about 20 min at each concentration. The leaf samples were then dried using a critical

point drier (Tousimis Samdri PVT B). The samples were then mounted vertically on stubs and coated with gold/palladium in a Hummer VI sputter coater. The leaf surface and leaf cross sections were then viewed using a Hitachi S-3400 scanning electron microscope operating at 5kV.

2.6.4 CO2 gas exchange measurements

CO_2 gas exchange rates were measured on fully expanded third leaves of 25-day-old NA and 75-day-old CA plants. At these ages, the plants were considered to be at comparable physiological stages of development based on growth kinetics. CO_2 gas exchange rates were measured by using the LI-COR portable infrared CO_2 gas analyzer (LI-6400 XRT Portable Photosynthesis System, LI-COR Biosciences, Lincoln, NE, USA). The apparent maximum quantum efficiency (Q) and the maximal photosynthetic capacity (A_{sat}) for the NA and CA plants were determined as the maximum initial slope of the linear portion of the light response curves and maximum light-saturated rates of photosynthesis respectively from light response curves using 12 irradiance values over the range of 0 to 1500 µmol photons m^{-2} s^{-1} PPFD. Light response curves were measured from high to low light intensity with 8 min of waiting time between the measurements. Maximum rate of photosynthetic electron transport for CO_2 gas exchange (J_{max}) was calculated by using computer software "Photosyn Assistant" (Dundee scientific, University of Dundee, UK). Rubisco-limited carboxylation efficiency (CE) was determined as the maximum initial slope of the linear portion of the CO_2 response curves and the CO_2- saturated carboxylation capacity was calculated from CO_2 response curves measured by supplying 11 different CO_2 concentrations over the range of 50 to 1200 µmol C mol^{-1} at a saturating irradiance. Respiration rates (R_{dark}) were measured in the dark. In addition, stomatal conductance and leaf transpiration rates were measured simultaneously with CO_2 gas exchange measurements. Leaf water use efficiency was

calculated as the rate of CO_2 assimilated divided by the stomatal conductance (A_{sat}/gs).

Stomatal density was estimated on both adaxial and abaxial leaf surfaces of NA and CA wheat and rye cultivars using a Leica microscope (Leica ATC™ 2000, Buffalo, NY, USA) equipped with a stage micrometer (1mm^2) at a magnification of 100X. Stomatal density for WT and the *BNCBF17* -overexpressing line of *Brassica napus* was estimated from micrographs of leaf surfaces obtained by scanning electron microscope as described above.

2.6.5 Room Temperature Chl a Fluorescence

Chl a fluorescence was measured concomitantly with CO_2 gas exchange on fully expanded third leaves using LI-COR portable photosynthesis system (LI-6400 XRT, LI-COR Biosciences, Lincoln, NE, USA). All measurements of Chl a fluorescence were carried out by using the standard fluorescence leaf chamber (2 cm^2). Prior to fluorescence measurements the leaves were dark-adapted for 20 min. Minimum fluorescence (F_o) was measured by illuminating dark-adapted leaves with a low irradiance measuring beam (PPFD < 1μmol photons m^{-2} s^{-1}) from a light emitting diode. Maximal fluorescence (F_m) was determined by application of second flash of light (PPFD >5000 μmol photons m^{-2} s^{-1}) with a pulse duration of 0.8 s. Afterwards, an actinic light (PPFD 1300 μmol photons m^{-2} s^{-1}) was applied. Superimposed on the actinic beam was another saturating light flash (PPFD > 5000 μmol photons m^{-2} s^{-1}; 0.8 s) applied repetitively at 20 s intervals to determine maximal fluorescence in the light-adapted leaf (F_m'). Light-adapted steady state fluorescence (F_s) was determined by measuring the level of fluorescence immediately before the saturating flash. Finally, minimal fluorescence (F_o') in the light-adapted leaf was measured by turning off the actinic light. This allowed us to calculate maximum photochemical efficiency

(F_v/F_m), photochemical quenching (qP), non-photochemical quenching (NPQ), excitation pressure (1-qP) and photosynthetic electron transport rate (ETR).

2.6.6 Carbon isotope discrimination (δ 13C)

Leaf samples were freeze-dried and ground into a fine powder using a ball mill. About 0.1 mg of homogenized fine powder was sealed into a 5 x 3.5 mm-sized tin capsule. Samples were combusted using Costech ECS 4010 elemental analyzer coupled to a Thermo Finnigan Delta V Plus isotope ratio mass spectrometer in continuous flow mode. The carbon isotope ratios ($R = {}^{13}C/{}^{12}C$) of the samples (R_{sample}) and standard ($R_{standard}$) were determined. R values were converted to $\delta^{13}C$ values (in ‰ or per mil) using the relationship: $\delta^{13}C$ (‰) = [$R_{sample}/R_{standard}$-1] x 1000. The δ ^{13}C values were calibrated to VPDB using NBS-22 (accepted value = -30.0 ‰) and IAEA - CH - 6 (accepted value: -10.5 ‰), to yield the accepted value of 1.95 ‰ for NBS-19 (Coplen, 1994).

2.6.7 Determination of total leaf protein and immunodetection

After each CO_2 gas exchange measurement, the fully expanded third leaves of 25-day-old NA and 75-day-old CA plants were harvested, immediately frozen in liquid N_2 and stored at -80°C. The frozen leaf samples were ground into a fine powder using liquid N_2 in a mortar and pestle. About 30 - 35 mg of ground leaf samples were added to 800 µl of cold (4°C) extraction buffer containing 1M Tris-HCl (pH 6.8), 10% (w/v) SDS, 15% (w/v) sucrose and 0.5M DTT. The samples were vortexed briefly, solubilized at 70°C for 10 min and centrifuged to remove debris. Total leaf protein concentrations of the supernatant were quantified using the RC-DC protein assay kit (Bio-Rad) according to the manufacturer's instructions. While quantifying the total leaf protein content, the addition of 1 µg of bovine serum albumin (Invitrogen) in the extraction buffer was used as an internal standard.

The extracted proteins were electrophoretically separated using NuPAGE Novex 10% (w/v) Bis-Tris precast, polyacrylamide gels (Invitrogen) with MES SDS running buffer (Invitrogen) in an XCell4 SureLock Midi Cell (Invitrogen) according to manufacturer's instructions. Samples were loaded for SDS-PAGE on an equal protein basis (8 µg protein per lane). Gels were electrophoresed at 80V for about 3 h. For immunodetection, separated polypeptides were electroblotted onto nitrocellulose membranes (0.2 µM pore size, Bio - Rad) in transfer buffer for 1 h at 100V as described by Gray *et al.*, (1996). The membranes were blocked with 5% (w/v) fat free, dried milk powder overnight at 4°C and then probed with primary antibodies raised against the target proteins; rbcL, cFBPase, Lhcb1, PsbA (D1) and PsaA at a dilution of 1:2000-5000. The blots were rinsed briefly and washed in TBS wash buffer at room temperature with agitation. The blots were probed with secondary antibody (anti-rabbit IgG Peroxidase antibody, Sigma-Aldrich) at a 1:10000-20000 dilutions for 1 h at room temperature with agitation. The blots were washed as described above and the target proteins were visualized with enhanced chemiluminesence immunodetection (ECL Detection Kit; GE Healthcare, UK) on X-ray film (Fujifilm, Fuji Corporation, Tokyo). Immunoblots were quantified by using a computer software program (Scion Image, Scion Corporation, Frederick, Maryland 21701, USA).

2.6.8 Analyses of gene expression

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out to assess the effects of cold acclimation on photosynthetic gene expression. The transcript levels for *rbcL*, *cFBPase*, *Lhcb1*, *PsbA* and *PsaA* were quantified by qRT-PCR. Total RNA was extracted from wheat leaves (200mg fresh weight) with Trizol reagent (Invitrogen). For qRT-PCR, 10 µg of total RNA was digested with DNASE I (Invitrogen) and half of the digested RNA was used for cDNA synthesis with random hexamers and Superscript Reverse Transcriptase (Invitrogen). The cDNA obtained

were diluted ten-fold in deionized water and 2 µl were used as template for real-time PCR. The polymerase chain reaction was performed with SYBR-Green PCR Mastermix (Invitrogen) and amplification was monitored on an ABI PRISM 7000 (Applied Biosystem). The 18S ribosomal gene was used as an internal standard for normalization of expression levels. The PCR amplification efficiency was 1.4 for all the genes tested. The primers used for the different genes are listed in Appendix S1 in Supporting Information.

2.6.9 Statistical analysis

In all experiments, 20 replicate pots with three plants per pot for each cultivar in either the NA or CA state were grown in a completely randomized design. Out of the 20 replicate pots, three pots for each cultivar at each growth condition were randomly selected for all photosynthetic measurements and biochemical analyses. Thus, all data are the averages of measurements made on nine different plants from three replicate pots.

Results were subjected to analysis of variance (ANOVA). When conducting ANOVA, growth parameters, leaf protein content, CO_2 gas exchange rates and photosynthetic characteristics were considered as dependent variables while the growth temperatures were considered as independent variables. Means were compared at the 5% level of significance ($P \leq 0.05$) by using the statistical package SPSS version 17.

2.7 ACKNOWLEDGMENTS

This work was supported, in part, by the Natural Sciences and Engineering Research Council (NSERC) and industrial and government partners, through the Green Crop Research Network (GCN). NPAH, FS, BG and FJL also acknowledge research support through their individual NSERC Discovery Grants. Scanning electron microscopy was carried out at the Biotron Experimental Climate Change Research Centre, The University of Western Ontario with the help of Richard Gardiner. Stable isotope analyses were performed in the Laboratory for Stable Isotope Science at the University of Western Ontario with the assistance of Kim Law.

Table 2.1: Effects of growth temperatures on growth characteristics of winter (cv Musketeer rye; cv Norstar wheat) and spring (cv SR4A rye; cv Katepwa wheat) rye and wheat cultivars grown at 20°C (NA) and 5°C (CA). Samples were collected from fully expanded third leaves of 25-day-old NA and 75-day-old CA plants. Data represent the mean of nine plants from three different pots ± SD. Significant differences of the means between the growth temperatures within each cultivar are indicated by the symbol * ($P \leq 0.05$). na = Not available.

Growth characteristics	Acclimation	Musketeer	Norstar	SR4A	Katepwa
Exponential growth rates	NA	0.223 ± 0.041	0.215 ± 0.024	0.229 ± 0.029	0.212 ± 0.036
	CA	0.072 ± 0.010*	0.069 ± 0.008*	0.075 ± 0.02*	0.065 ± 0.014*
Leaf water content (%)	NA	87 ± 3	88 ± 3	88 ± 4	86 ± 3
	CA	67 ± 6*	71 ± 8*	82 ± 5	83 ± 4
Total shoot dry mass (mg plant $^{-1}$)	NA	266 ± 38	219 ± 19	310 ± 55	202 ± 23
	CA	228 ± 27	181 ± 32	271 ± 41	136 ± 20*
Dry weight: fresh weight ratio	NA	0.13 ± 0.03	0.12 ± 0.03	0.12 ± 0.02	0.14 ± 0.03
	CA	0.33 ± 0.05*	0.29 ± 0.06*	0.18 ± 0.02*	0.17 ± 0.04
Shoot : root ratio	NA	3.26 ± 0.24	3.31 ± 0.23	4.51± 0.46	2.90 ± 0.51

	CA	4.02 ± 0.41*	3.43 ± 0.30	4.85 ± 0.72	2.29 ± 0.19
Total chlorophyll	NA	599 ± 58	645 ± 46	602 ± 45	531 ± 34
($mg\ m^{-2}$)	CA	861 ± 79*	784 ± 38*	561 ± 24	480 ± 59
Chl a/chl b	NA	2.65 ± 0.18	2.60 ± 0.19	2.81± 0.13	2.58 ± 0.36
	CA	2.63 ± 0.25	2.45 ± 0.11	3.08 ± 0.20	2.21 ± 0.29
Total leaf protein ($g\ m^{-2}$ leaf area)	NA	2.70 ± 0.46	2.32 ± 0.31	3.33 ± 0.54	2.89 ± 0.45
	CA	12.65 ± 2.20*	9.84 ± 1.60*	4.01± 0.51	3.27 ± 0.42
Protein/Chl Ratio	NA	4.5 ± 0.8	3.6 ± 0.2	5.5 ± 0.8	5.4 ± 0.4
	CA	14.7 ± 1.2*	12.5 ± 1.5*	7.1 ± 0.9	6.8 ± 1.1
$\delta^{13}C$ (‰, VPDB)	NA	-29.5 ± 0.2	na	-29.2 ± 0.4	na
	CA	-27.2 ± 0.4*	na	-29.1 ± 0.3	na

Table 2.2: Light-saturated rates of net CO2 assimilation (Asat) and dark respiratory rates (Rdark) for winter and spring wheat and rye cultivars grown at either 20°C (NA) or 5°C (CA) and at ambient CO2 conditions. Photosynthetic and dark respiratory rates were measured on attached, fully developed third leaves of 25-day-old NA and 75-day-old CA plants at measuring temperatures of either 20 °C or 5 °C and at ambient CO2. Data represent the mean of nine plants from three different pots ± SD. Significant differences among the means within each cultivar are indicated by the superscripted letters ($P \leq 0.05$).

		A_{sat} (µmol CO_2 fixed $m^{-2} s^{-1}$)		R_{dark} (µmol CO_2 evolved $m^{-2} s^{-1}$)	
		Measuring temperatures (°C)		Measuring temperatures (°C)	
Cultivars	Acclimation	20	5	20	5
Musketeer	NA	18.1 ± 1.6^{b}	11.8 ± 1.2^{a}	3.24 ± 0.14^{b}	2.7 ± 0.21^{a}
	CA	25.4 ± 1.5^{c}	20.9 ± 1.8^{b}	4.06 ± 0.18^{c}	3.88 ± 0.23^{c}
Norstar	NA	16.8 ± 0.9^{b}	9.9 ± 1.6^{a}	2.28 ± 0.11^{b}	1.71 ± 0.15^{a}
	CA	20.5 ± 1.8^{c}	17.7 ± 1.3^{bc}	2.96 ± 0.23^{c}	2.85 ± 0.18^{c}
SR 4A	NA	20.4 ± 1.7^{b}	12.6 ± 1.0^{a}	3.05 ± 0.12^{b}	2.56 ± 0.19^{a}
	CA	14.6 ± 1.5^{a}	13.9 ± 1.4^{a}	3.59 ± 0.24^{c}	3.43 ± 0.13^{c}
Katepwa	NA	17.6 ± 1.1^{c}	9.2 ± 1.2^{a}	2.07 ± 0.24^{b}	1.52 ± 0.15^{a}
	CA	12.1 ± 0.8^{b}	11.3 ± 1.9^{ab}	2.55 ± 0.31^{b}	2.35 ± 0.25^{b}

Table 2.3: Light-saturated rates of maximum electron transport (μmol e- m-2 s-1) for Chl a fluorescence (ETR) and for CO2 gas exchange (Jmax), the initial linear slope of the light response curves (the apparent maximum quantum efficiency, Q, CO2/photon), the initial linear slope of the light-saturated A/Ci response curves (carboxylation efficiency, CE, CO2 m-2 s-1/ mol-1 CO2), for winter and spring cultivars. The measurements were carried out on attached, fully developed third leaves of 25-day-old NA and 75-day-old CA plants at measuring temperatures of either 20° or 5°C and at ambient CO2. Each point represents the mean of nine plants from three different pots ± SD. Significant differences among the means within each cultivar are indicated by the superscripted letters ($P \leq 0.05$).

Cultivars	Acclimation	Temperature (°C)	ETR	J_{max}	Q	CE
Musketeer	NA	20	166 ± 12^{b}	137 ± 8^{b}	0.048 ± 0.006^{b}	0.083 ± 0.007^{ab}
		5	122 ± 17^{a}	109 ± 14^{a}	0.033 ± 0.004^{a}	0.073 ± 0.005^{a}
	CA	20	235 ± 25^{c}	178 ± 21^{c}	0.059 ± 0.004^{c}	0.093 ± 0.006^{b}
		5	223 ± 28^{c}	162 ± 11^{c}	0.053 ± 0.008^{bc}	0.091 ± 0.016^{b}
Norstar	NA	20	157 ± 10^{b}	139 ± 10^{a}	0.045 ± 0.006^{b}	0.078 ± 0.012^{ab}
		5	128 ± 11^{a}	118 ± 16^{a}	0.034 ± 0.003^{a}	0.065 ± 0.008^{a}
	CA	20	202 ± 18^{b}	172 ± 13^{b}	0.049 ± 0.007^{b}	0.088 ± 0.006^{b}
		5	208 ± 17^{b}	167 ± 9^{b}	0.047 ± 0.005^{b}	0.084 ± 0.009^{b}
SR 4A	NA	20	197 ± 15^{b}	142 ± 17^{b}	0.058 ± 0.004^{b}	0.094 ± 0.007^{b}
		5	142 ± 9^{a}	115 ± 9^{a}	0.035 ± 0.004^{a}	0.079 ± 0.011^{ab}
	CA	20	161 ± 13^{a}	109 ± 11^{a}	0.041 ± 0.005^{a}	0.064 ± 0.008^{a}
		5	148 ± 9^{a}	106 ± 14^{a}	0.045 ± 0.007^{a}	0.069 ± 0.01^{a}
Katepwa	NA	20	182 ± 20^{b}	148 ± 13^{b}	0.052 ± 0.009^{b}	0.087 ± 0.011^{b}
		5	116 ± 15^{a}	108 ± 15^{a}	0.029 ± 0.005^{a}	0.060 ± 0.008^{a}
	CA	20	127 ± 8^{a}	115 ± 10^{a}	0.037 ± 0.004^{a}	0.052 ± 0.005^{a}
		5	122 ± 13^{a}	113 ± 14^{a}	0.044 ± 0.006^{b}	0.061 ± 0.008^{a}

Table 2.4: Stomatal conductance (gs, mol m-2 s-1), transpiration rates (mmol H2O m-2 s-1) and leaf water use efficiency (WUE, Asat/gs) of fully developed third leaves of winter and spring wheat and rye cultivars grown at either 20°C (NA) or 5°C (CA) and at ambient CO2. Data represent the mean of nine plants from three different pots ± SD. Significant differences among the means within each cultivar are indicated by the superscripted letters ($P \leq 0.05$)

Cultivars	Acclimation state	Measuring temperatures	Stomatal conductance	Transpiration	WUE
Musketeer	NA	20	0.61 ± 0.09^{c}	2.65 ± 0.30^{b}	29.7 ± 1.9^{a}
		5	0.40 ± 0.05^{b}	2.23 ± 0.19^{b}	29.5 ± 2.6^{a}
	CA	20	0.25 ± 0.03^{a}	1.68 ± 0.11^{a}	98 ± 10.4^{c}
		5	0.27 ± 0.04^{a}	1.79 ± 0.12^{a}	77.4 ± 5.1^{b}
Norstar	NA	20	0.68 ± 0.11^{c}	3.42 ± 0.24^{c}	24.7 ± 3.2^{a}
		5	0.36 ± 0.06^{b}	2.86 ± 0.16^{b}	27.5 ± 1.3^{a}
	CA	20	0.22 ± 0.03^{a}	1.93 ± 0.18^{a}	93.2 ± 8.7^{c}
		5	0.25 ± 0.05^{ab}	1.76 ± 0.41^{a}	70.8 ± 5.3^{b}
SR4A	NA	20	0.81 ± 0.13^{b}	3.09 ± 0.22^{b}	25.2 ± 1.8^{b}
		5	0.73 ± 0.10^{ab}	2.51 ± 0.15^{a}	17.2 ± 2.5^{a}
	CA	20	0.61 ± 0.04^{a}	2.44 ± 0.32^{a}	23.9 ± 3.2^{ab}
		5	0.56 ± 0.06^{a}	2.39 ± 0.29^{a}	24.8 ± 2.2^{b}
Katepwa	NA	20	0.54 ± 0.07^{b}	3.24 ± 0.49^{b}	32.6 ± 2.9^{b}
		5	0.62 ± 0.11^{b}	2.30 ± 0.31^{a}	14.8 ± 1.2^{a}
	CA	20	0.35 ± 0.05^{a}	2.39 ± 0.16^{a}	34.6 ± 2.1^{b}
		5	0.32 ± 0.05^{a}	2.56 ± 0.17^{a}	35.3 ± 3.1^{b}

Table 2.5: Morphological and physiological characteristics of wild type (WT) and BNCBF17 -overexpressing Brassica transgenic line (BNCBF17) grown at 20°C and at ambient CO2 conditions. All measurements were carried out on fully developed third leaves of three-week-old WT and four-week-old BNCBF17 at measuring temperatures of 20°C and at ambient CO2. Data represent the mean of three plants from three different pots ± SD. Significant differences of the means between the two lines are indicated by the symbol * ($P \leq 0.05$).

Morphological and physiological characteristics		Genotypes	
		Wild Type	*BNCBF17*
Specific leaf weight (g dry weight m^{-2} leaf area)		24 ± 4	41 ± 6*
Total Chlorophyll (mg m^{-2})		367 ± 48	984 ± 74*
Chlorophyll a/Chlorophyll b ratio		3.3 ± 0.12	2.8 ± 0.19*
Q (CO_2/photon)		0.051 ± 0.003	0.061 ± 0.004*
Carboxylation efficiency (CE - CO_2 m^{-2} s^{-1}/mol^{-1} CO_2)		0.097 ± 0.012	0.115 ± 0.017
$\delta^{13}C$		-31.1 ± 0.3	-29.6 ± 0.4*
R_{dark} (µmol CO_2 evolved m^{-2} s^{-1})		- 3.43 ± 1.18	- 2.58 ± 0.38
Stomatal conductance gs (mol m^{-2} s^{-1})		0.29 ± 0.04	0.32 ± 0.04
Stomatal frequency (Stomates mm^{-2} leaf area)	Adaxial	126 ± 15	160 ± 28
	Abaxial	375 ± 19	345 ± 37
Transpiration (mmol H_2O m^{-2} s^{-1})		1.96 ± 0.93	2.14 ± 0.34
Water use efficiency (WUE - A_{sat}/gs)		56.6 ± 4	72.6 ± 6*

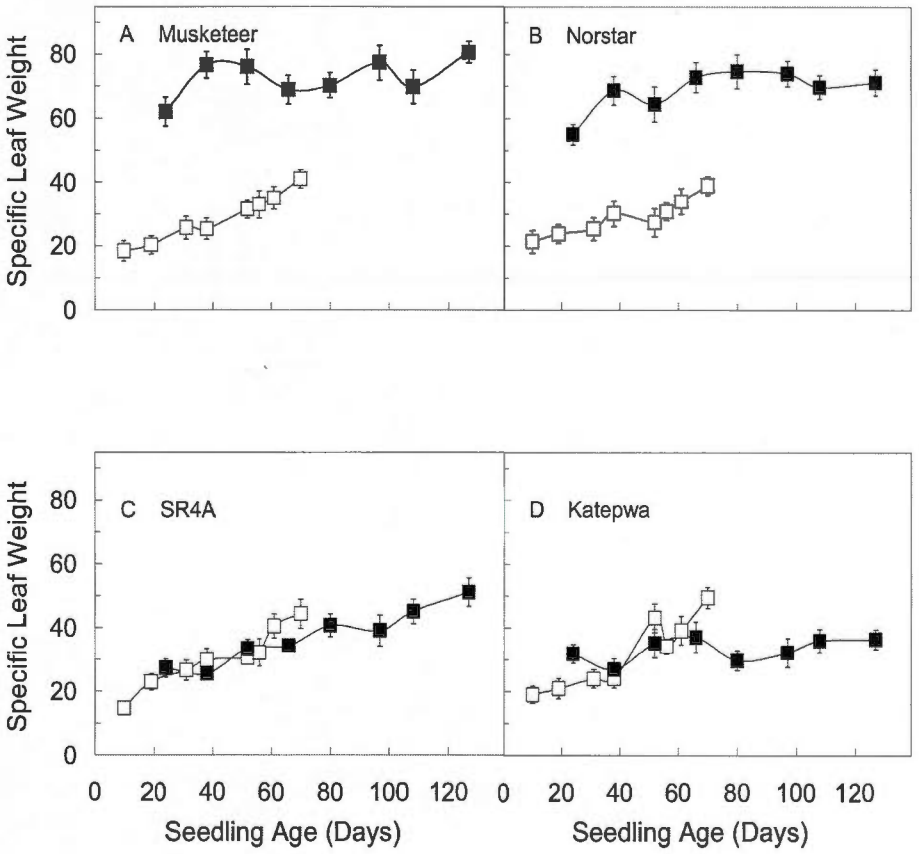

Figure 2.1: Specific leaf weight (dry weight g m-2 leaf area) for winter (cv Musketeer rye, cv Norstar wheat) and spring (cv SR4A rye, cv Katepwa wheat) rye and wheat cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Each point represents the mean of nine plants from three different pots. Bars represent SD.

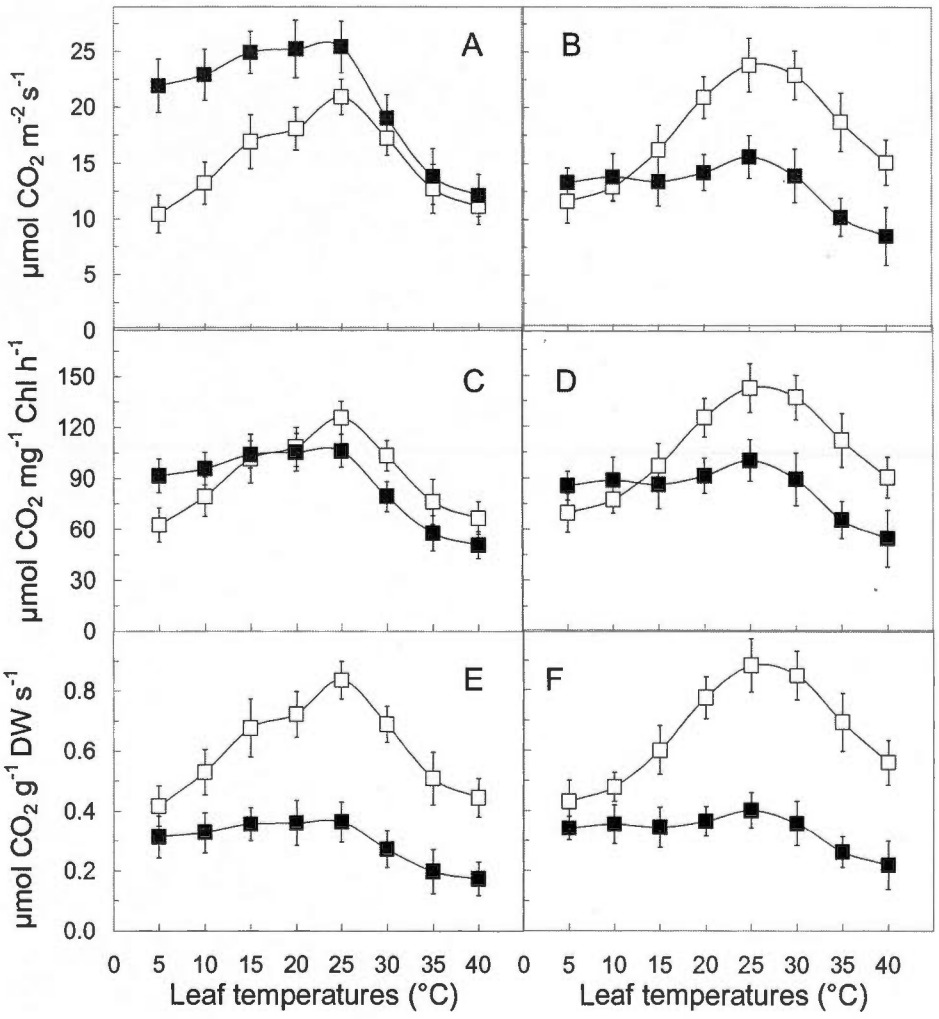

Figure 2.2: Temperature response curves of light-saturated net CO2 assimilation expressed on either leaf area (A, B) or leaf chlorophyll (C, D) or leaf dry weight (E, F) basis for Musketeer winter (A, C, E) and SR4A spring rye (B, D, F) cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Photosynthetic rates for both NA and CA plants were measured on attached, fully developed third leaves at varying leaf temperatures ranging from 5°C to 40°C and at ambient CO_2. Each point represents the mean of nine plants from three different pots. Bars represent standard deviation.

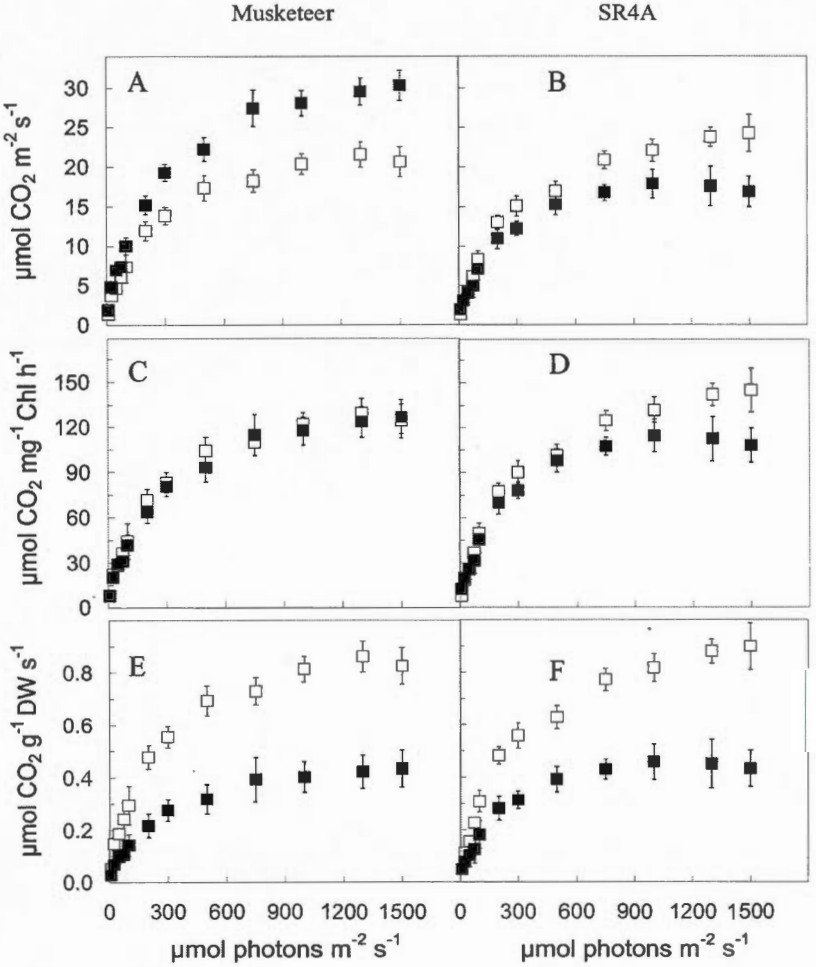

Figure 2.3: Light response curves of gross CO2 assimilation expressed on either leaf area (A, B) or leaf chlorophyll (C, D) or leaf dry weight (E, F) basis for Musketeer winter (A, C, E) and SR4A spring rye (B, D, F) cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Photosynthetic rates for both NA and CA plants were measured on attached, fully developed third leaves at 20°C. Each point represents the mean of nine plants from three different pots. Bars represent SD.

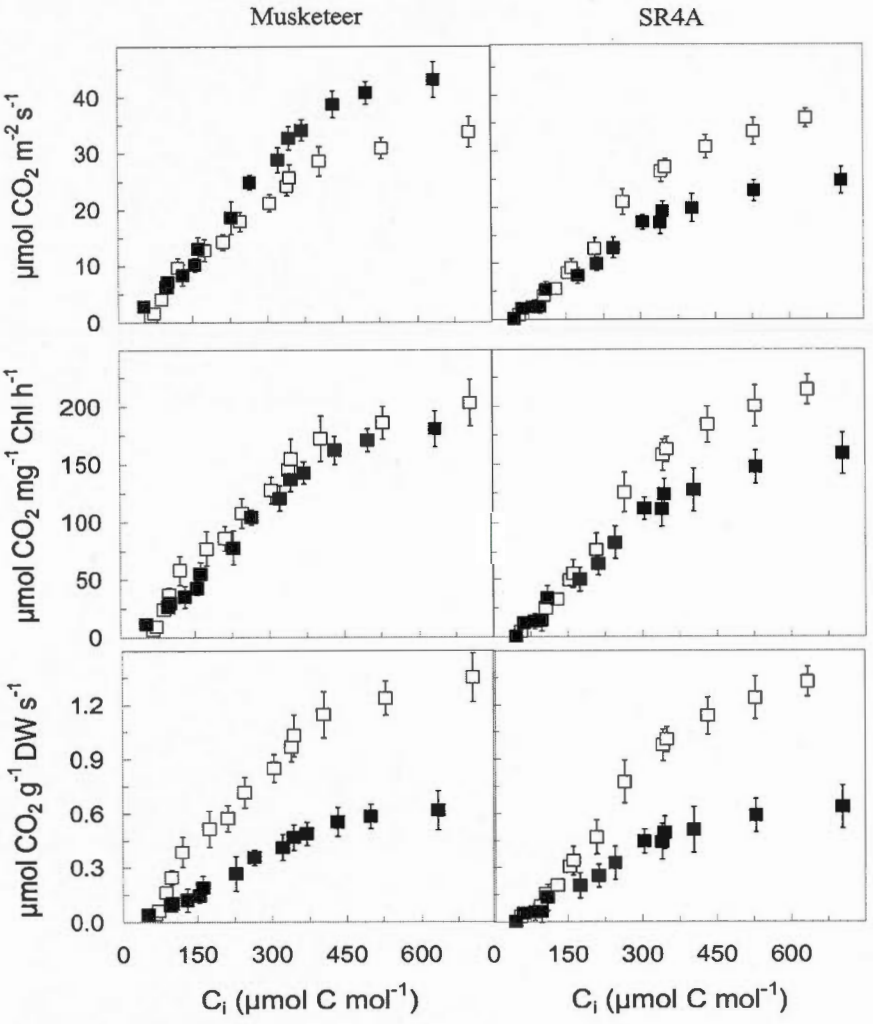

Figure 2.4: Light-saturated rates of net CO2 assimilation versus internal CO2 concentrations (Ci) expressed on either leaf area (A, B) or leaf chlorophyll (C, D) or leaf dry weight (E, F) basis for Musketeer winter (A, C, E) and SR4A spring rye (B, D, F) cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Photosynthetic rates for both NA and CA plants were measured on attached, fully developed third leaves at 20°C. Each point represents the mean of nine plants from three different pots. Bars represent SD.

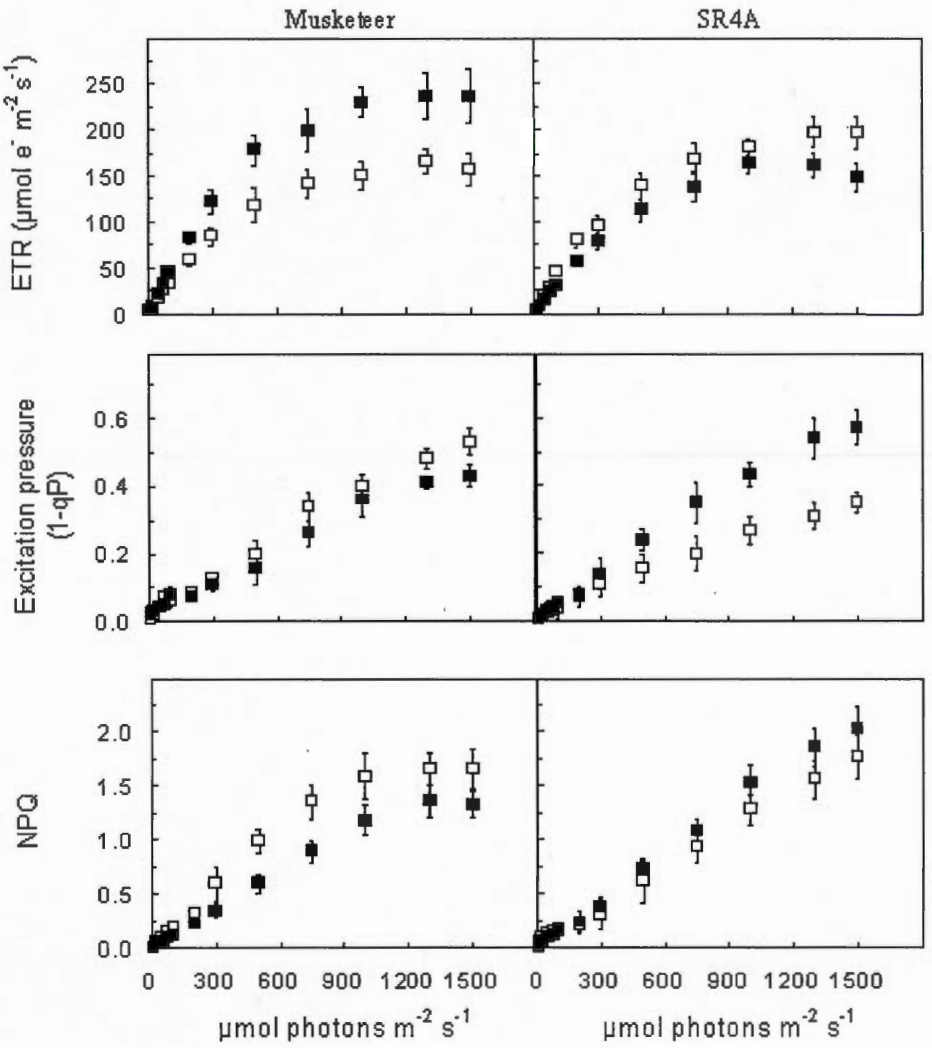

Figure 2.5: Light response curves of electron transport rates (ETR; A, B), excitation pressures (1-qP; C, D) and non photochemical quenching of excess energy (NPQ; E, F) for Musketeer winter (A, C, E) and SR4A spring rye (B, D, F) cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Measurements were carried out at 20°C for both NA and CA plants. Each point represents the mean of nine plants from three different pots. Bars represent SD.

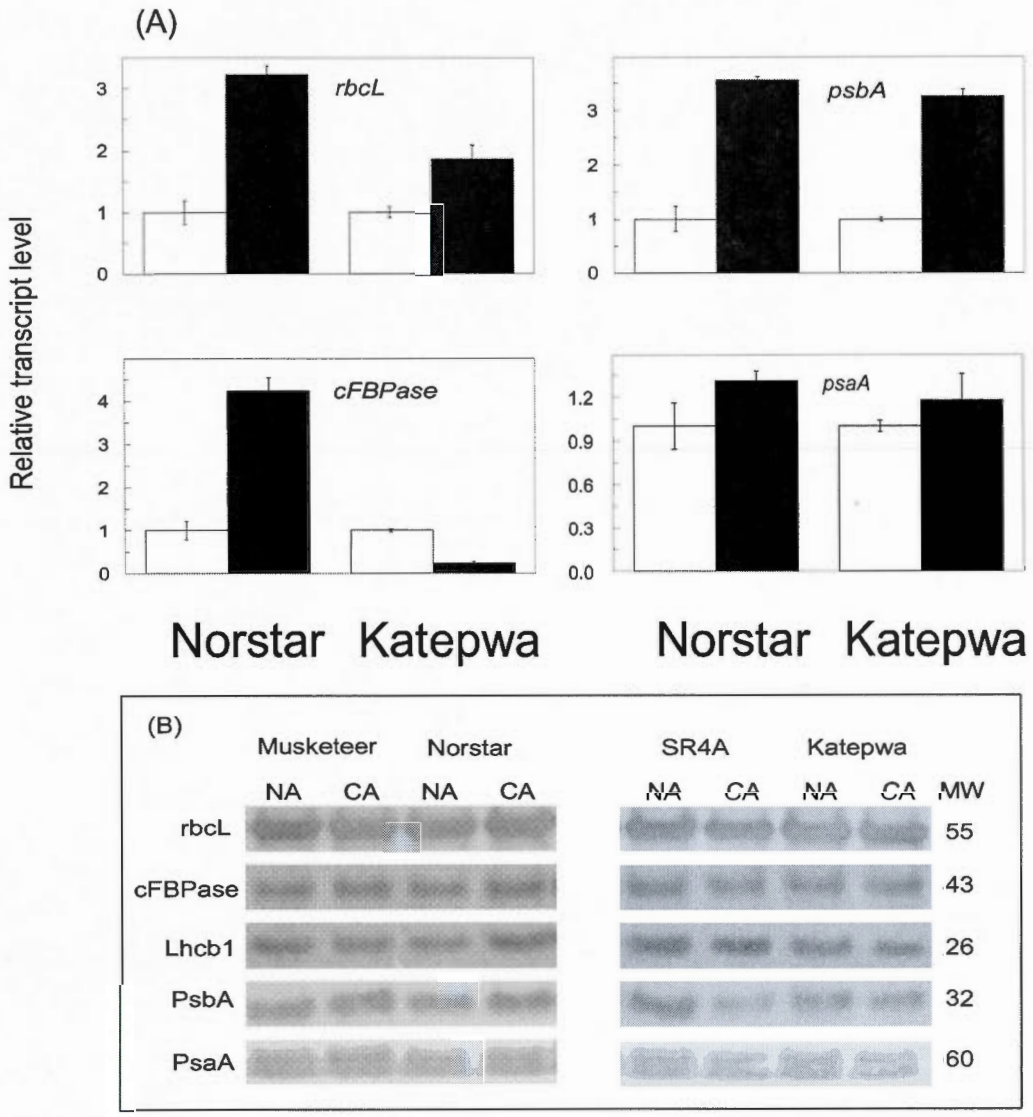

Figure 2.6: (A) Relative transcript levels of rbcL, cFBPase, lhcb1, psbA, and psaA isolated from Norstar winter and Katepwa spring wheat grown at either 20°C (□) or 5°C (■) and at ambient CO2. Samples were collected from fully developed third leaves of 25-d-old NA and 75-d-old CA plants. 18S gene was used as an internal control. (B) Immunoblot analysis of SDS – PAGE probed with antibodies raised against: rbcL, cFBPase, Lhcb1, PsbA and PsaA isolated from winter and spring rye and wheat cultivars grown at either 20°C (NA) or 5°C (CA) and at ambient CO2. Samples were collected from fully developed third leaves of 25-day-old NA and 75-day-old CA plants. Lanes of SDS – PAGE were loaded on equal

protein basis (8 μg protein/lane). The bovine serum albumin (1μg on each lane) was used as an internal control. Numbers on the right indicate molecular weight (MW, kDa) of markers.

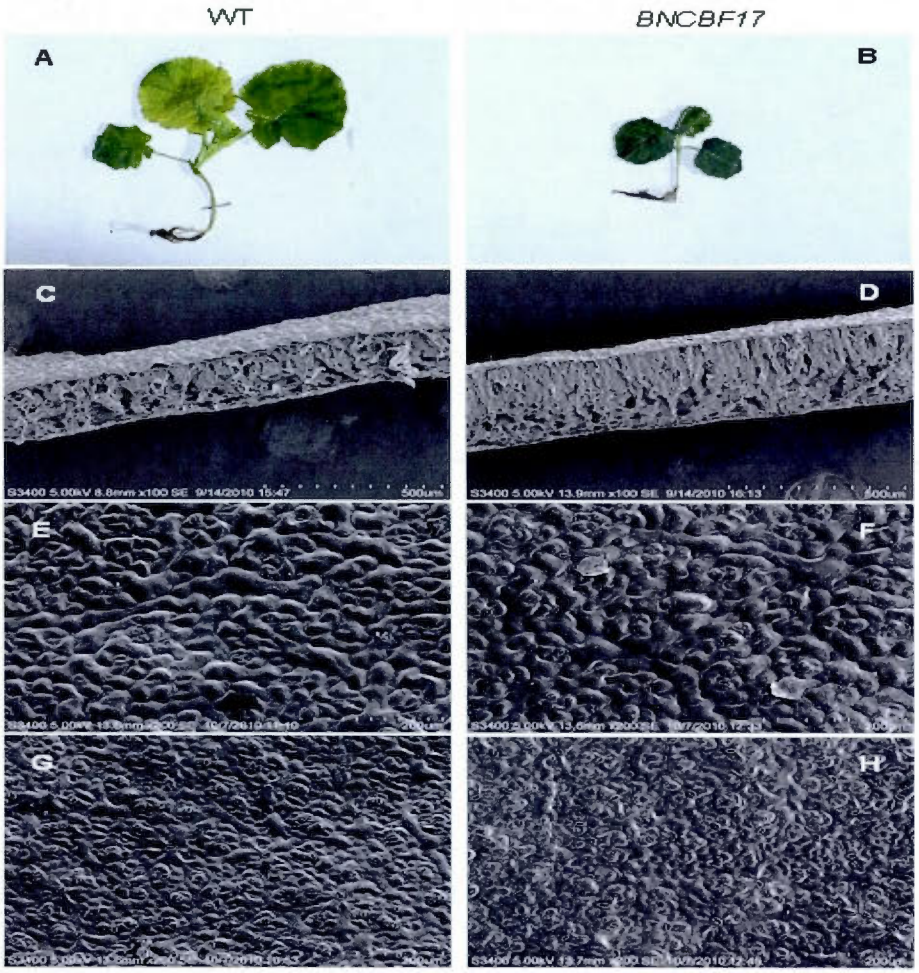

Figure 2.7: Effects of BNCBF17-overexpression on plant morphology, growth habit and leaf anatomy of two Brassica lines. Photographs for 3-week-old wild type (WT, A) and four-week-old BNCBF17-overexpressing Brassica transgenic lines (BNCBF17, B) grown at 20°C and at ambient CO2 conditions. Leaf cross sections for WT (C) and BNCBF17 (D). Upper leaf surfaces for WT (E) and BNCBF17 (F). Lower leaf surfaces for WT (G) and BNCBF17 (H). The images for leaf cross sections and leaf surfaces were taken from fully developed third leaves of three-week-old WT and four-week-old BNCBF17-overexpressing plants using SEM.

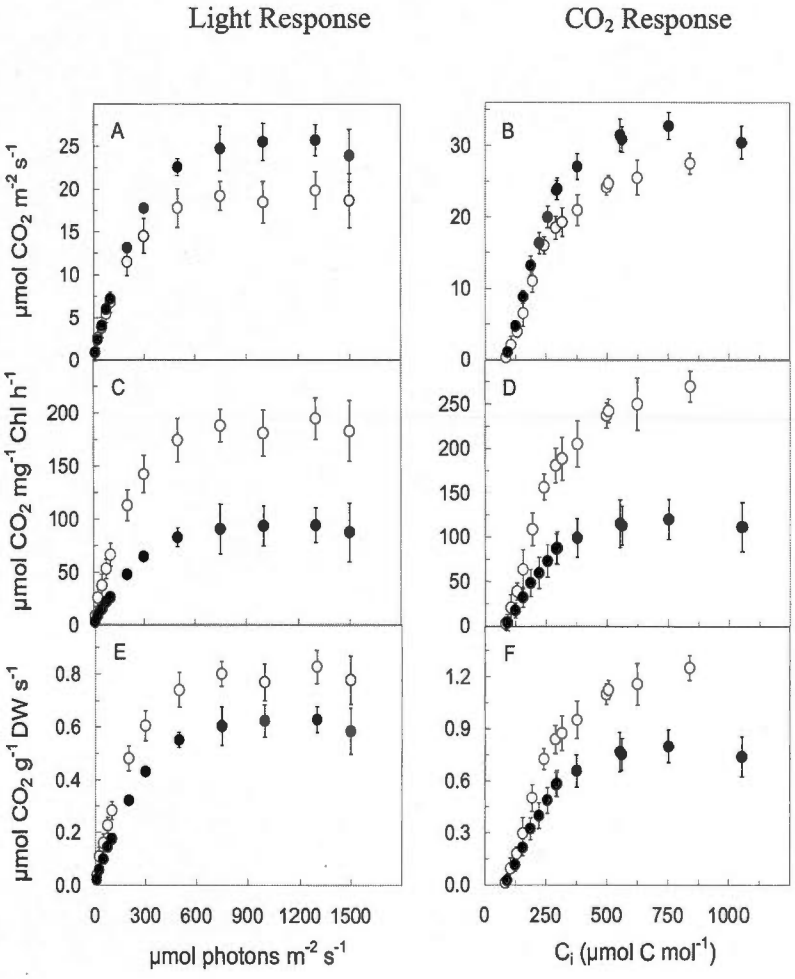

Figure 2.8: Light response curves of gross CO2 assimilation (A, C, E) and light-saturated rates of net CO2 assimilation versus internal CO2 concentrations (B, D, F) expressed on either leaf area (A, B) or leaf chlorophyll (C, D) or leaf dry weight (E, F) basis for WT (O) and BNCBF17-overexpressing Brassica transgenic lines (●) grown at 20°C and at ambient CO2 conditions. Photosynthetic rates were measured on fully developed third leaves of three-week-old WT and four-week-old *BNCBF17*-overexpressing plants at measuring temperature of 20°C and at ambient CO_2. Each point represents the mean of three plants from three different pots. Bars represent SD.

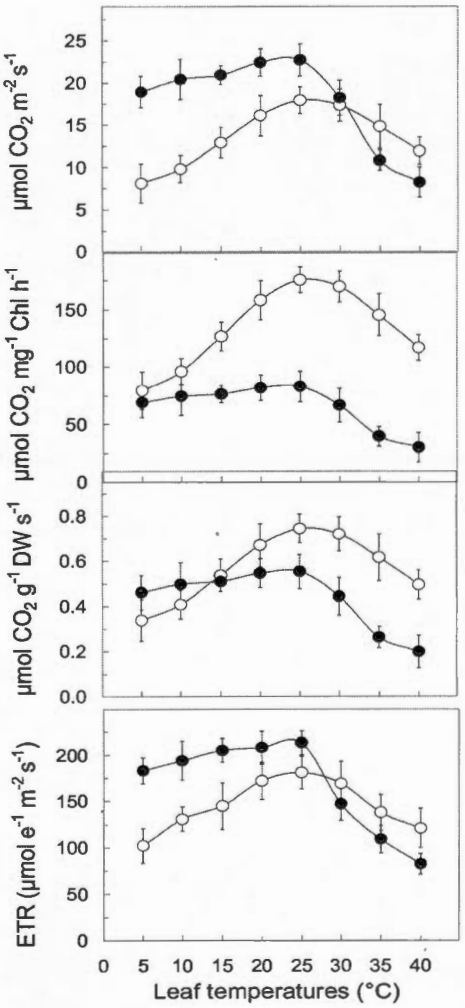

Figure 2.9: Temperature response curves of light-saturated net CO2 assimilation expressed on either leaf area (A) or leaf chlorophyll (B) or leaf dry weight (C) basis. (D) Temperature response curves for light-saturated electron transport rates (ETR) measured on leaf area basis for WT (O) and BNCBF17 transgenic line (●) grown at 20°C and at ambient CO2. Measurements were carried out on fully developed third leaves at varying leaf temperatures ranging from 5° to 40°C at ambient CO2. Each point represents the mean of three plants from three different pots. Bars represent SD.

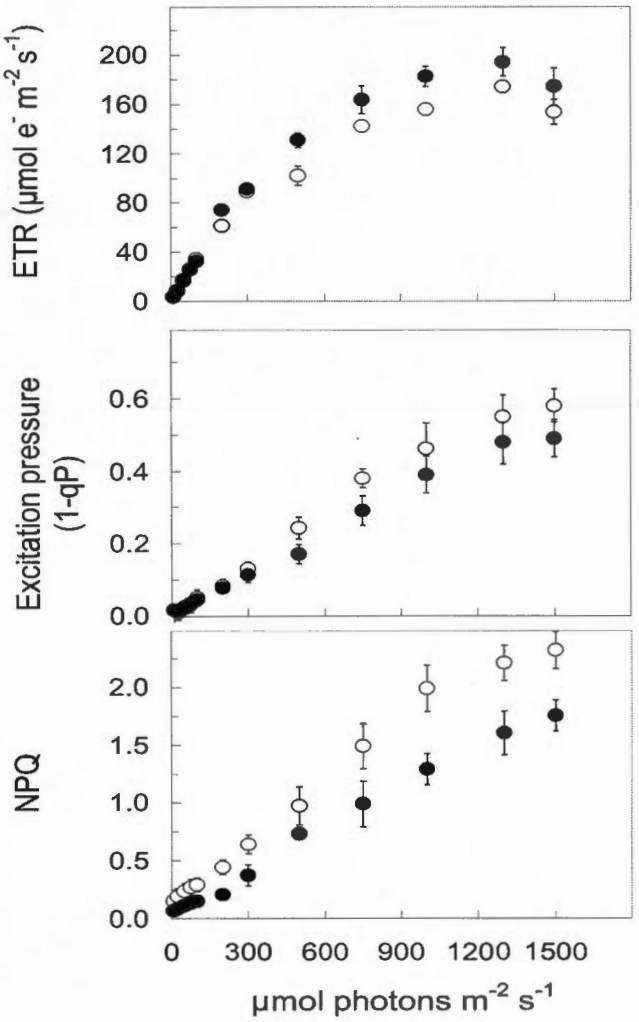

Figure 2.10: Light response curves of electron transport rates (ETR; A), excitation pressures (1-qP; B) and non photochemical quenching of excess energy (NPQ; C) for WT (O) and BNCBF17-overexpressing Brassica transgenic lines (●) grown at 20°C and at ambient CO2 conditions. Measurements were carried out at 20°C for both WT and transgenic lines. Each point represents the mean of three plants from three different pots. Bars represent SD.

2.8 SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table 2.S1: List of the primers used to analyze the transcript levels of major photosynthetic enzymes and components of photosynthetic electron transport in Norstar winter and Katepwa wheat cultivars.

Genes	Sequences
rbcL	5'-CTA CGC GGT GGA CTT GAT TT-3'
	3'-ATT TCA CCA GTT TCG GCT TG-5'
cFBPase	5'-AAC AAG AAC GAG GGA GGG ATA C-3'
	3'-TCC GCA TCA CAA GAA AAG G-5'
Lhcb1	5'-CGT CCT TCG GAC AAA TAT GC-3'
	3'-TAA TGA CAT GGG CCA GCA AG-5'
PsbA	5'-GTG GCT GCT CAC GGT TAT TT-3'
	3'-CCA AGC AGC CAA GAA GAA GT-5'
PsaA	5'-GGA AAA TGC AGT CGG ATG TT-3'
	3'-AGA AAT CTC GAA GCC AAC CA-5'

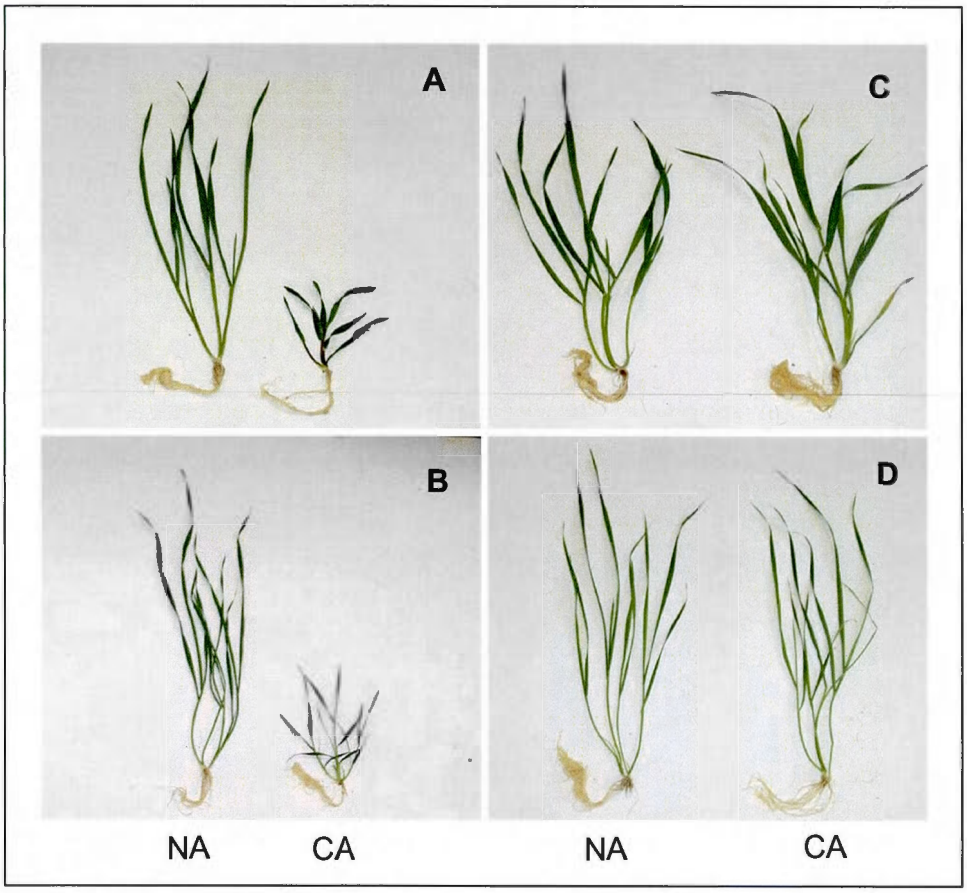

Figure 2.S1: Effects of growth temperatures on plant morphology and growth habit of winter (cv Musketeer rye, A; cv Norstar wheat, B) versus spring cultivars (cv SR4A rye, C; cv Katepwa wheat, D). Plants were grown at 20°C (NA) and 5°C (CA). Photographs were taken from 25-d-old NA and 75-d-old CA plants. The photographs for NA and CA plants of each cultivar were taken together.

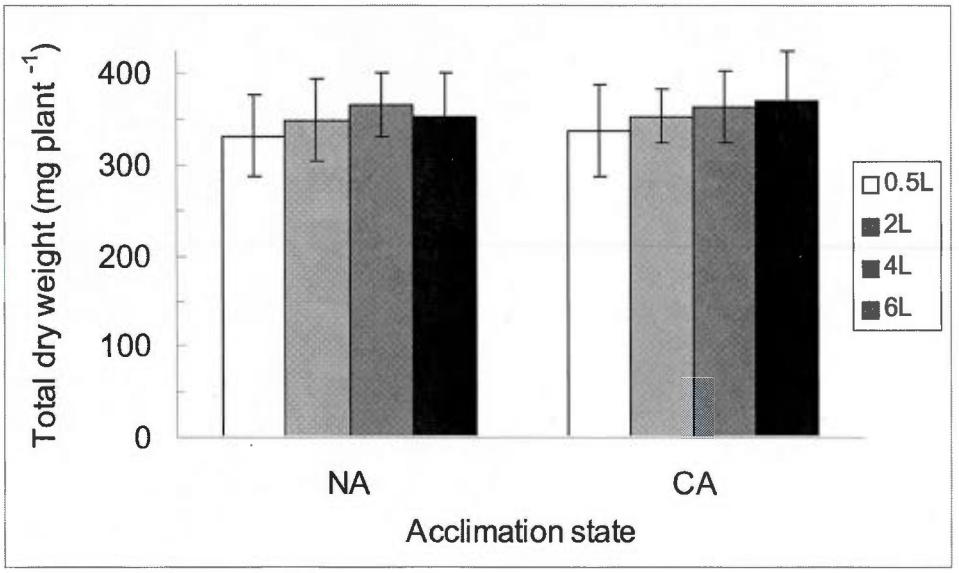

Figure 2.S2: Effect of pot size on plant growth. Musketeer winter rye was grown in 0.5L, 2L, 4L and 6L-sized pots at either 20°C (NA) or 5°C (CA) and at ambient CO2. The shoot and root samples were harvested from 25-d-old NA and 75-d-old CA plants. Each point represents the mean of nine plants from three different pots. Bars represent SD.

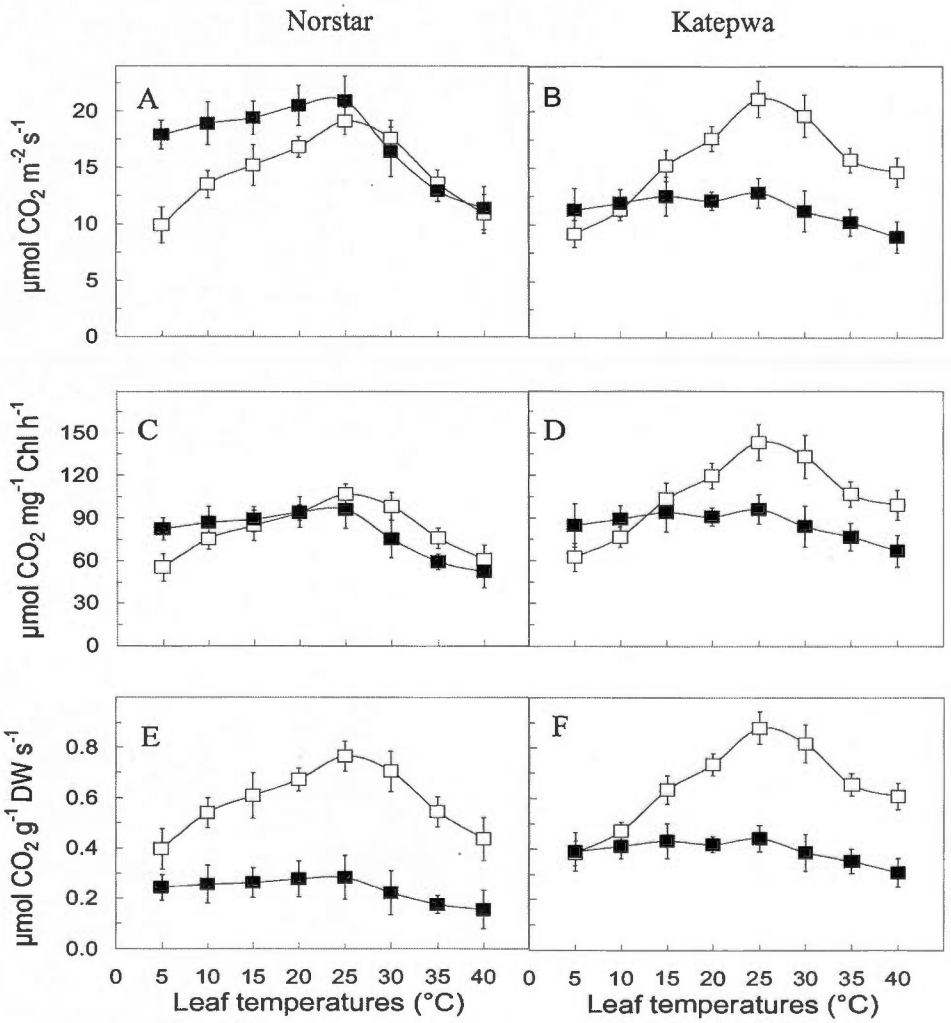

Figure 2.S3: Temperature response curves of light-saturated net CO2 assimilation expressed on either leaf area (A, B) or leaf chlorophyll (C, D) or leaf dry weight (E, F) basis for Norstar winter (A, C, E) and Katepwa spring wheat (B, D, F) cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Photosynthetic rates for both NA and CA plants were measured on attached, fully developed third leaves at varying leaf temperatures ranging from 5° to 40°C and at ambient CO_2. Each point represents the mean of nine plants from three different pots. Bars represent standard deviation.

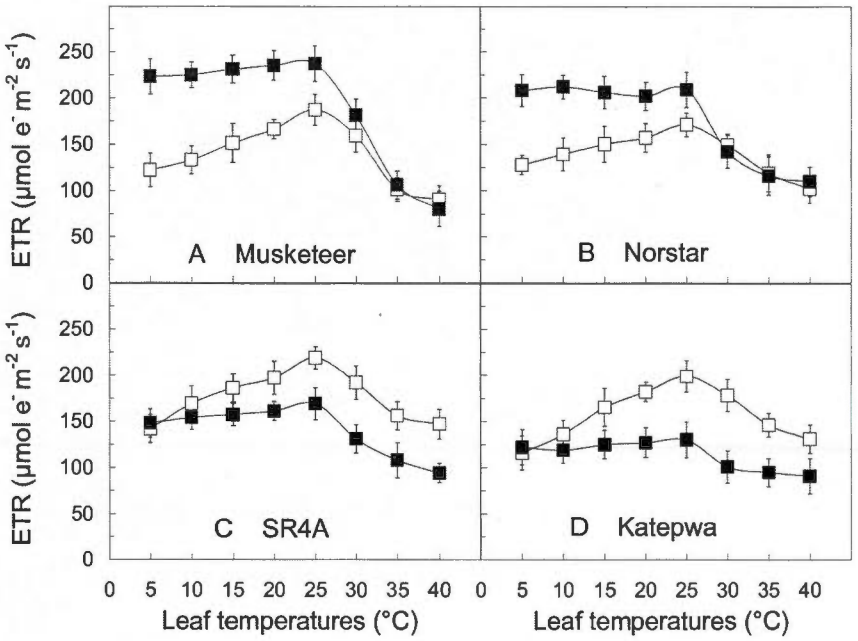

Figure 2.S4: Temperature response curves of light-saturated rates of electron transport (ETR) measured on a leaf area basis for winter (cv Musketeer rye, cv Norstar wheat) and spring (cv SR4A rye, cv Katepwa wheat) rye and wheat cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Measurements were carried out on fully developed third leaves at varying leaf temperatures ranging from 5° to 40°C at ambient CO_2. Each point represents the mean of three plants from three different pots. Bars represent SD.

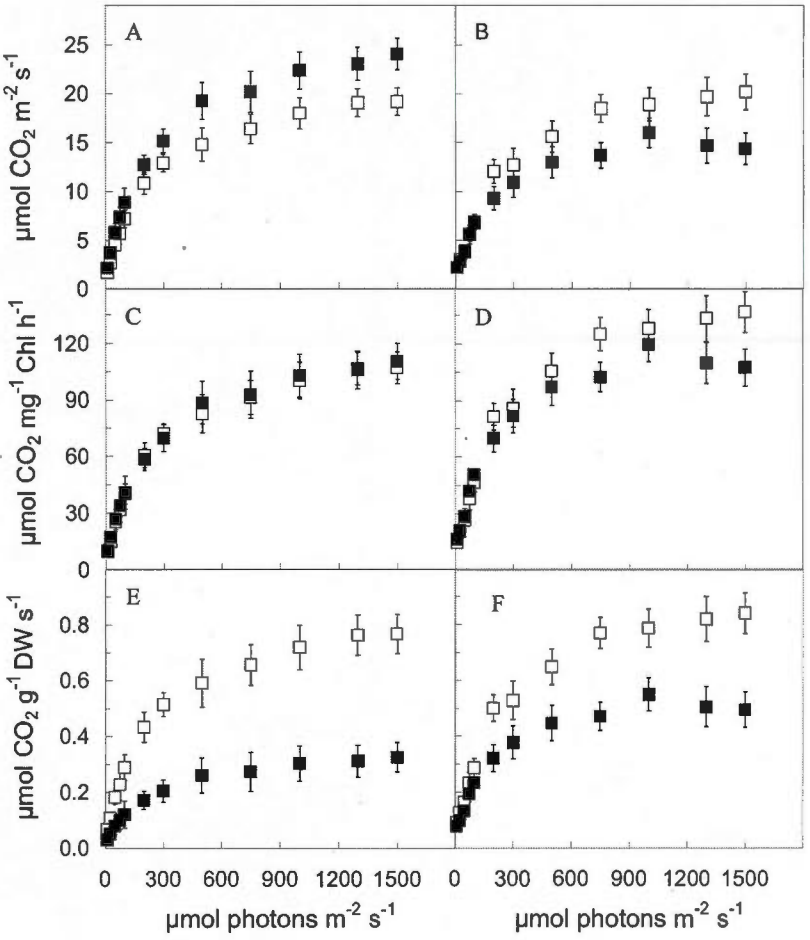

Figure 2.S5: Light response curves of gross CO2 assimilation expressed on either leaf area (A, B) or leaf chlorophyll (C, D) or leaf dry weight (E, F) basis for Norstar winter (A, C, E) and Katepwa spring wheat (B, D, F) cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Photosynthetic rates for both NA and CA plants were measured on attached, fully developed third leaves at 20°C. Each point represents the mean of nine plants from three different pots. Bars represent SD.

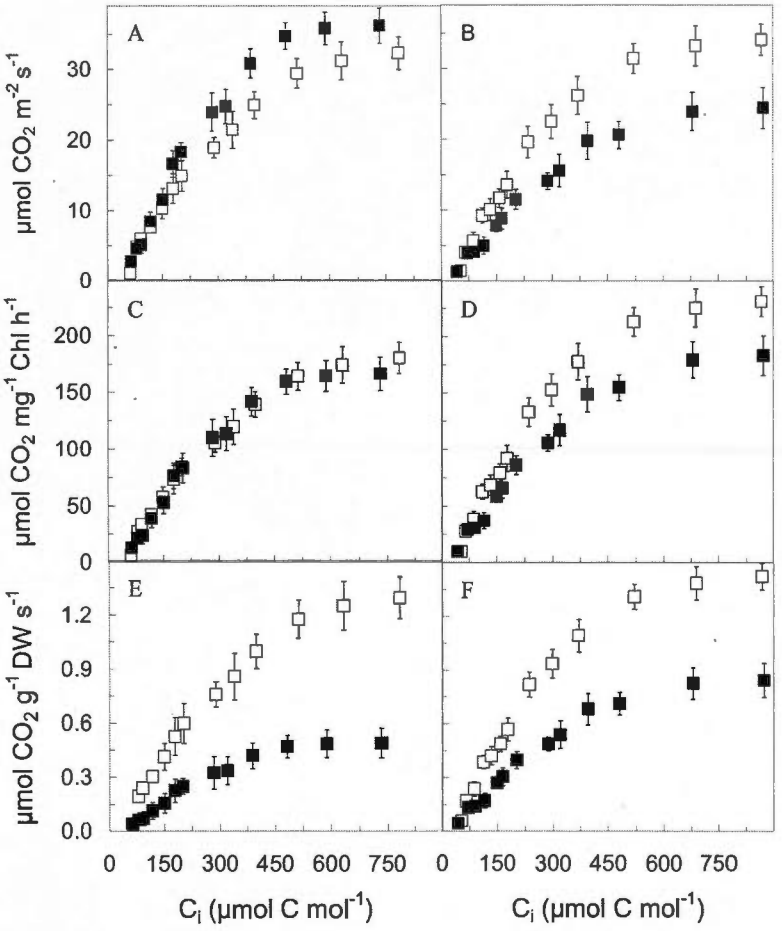

Figure 2.S6: Light-saturated rates of net CO2 assimilation versus internal CO2 concentrations (Ci) expressed on either leaf area (A, B) or leaf chlorophyll (C, D) or leaf dry weight (E, F) basis for Norstar winter (A, C, E) and Katepwa spring wheat (B, D, F) cultivars grown at either 20°C (□) or 5°C (■) and at ambient CO2. Photosynthetic rates for both NA and CA plants were measured on attached, fully developed third leaves at 20°C. Each point represents the mean of nine plants from three different pots. Bars represent SD.

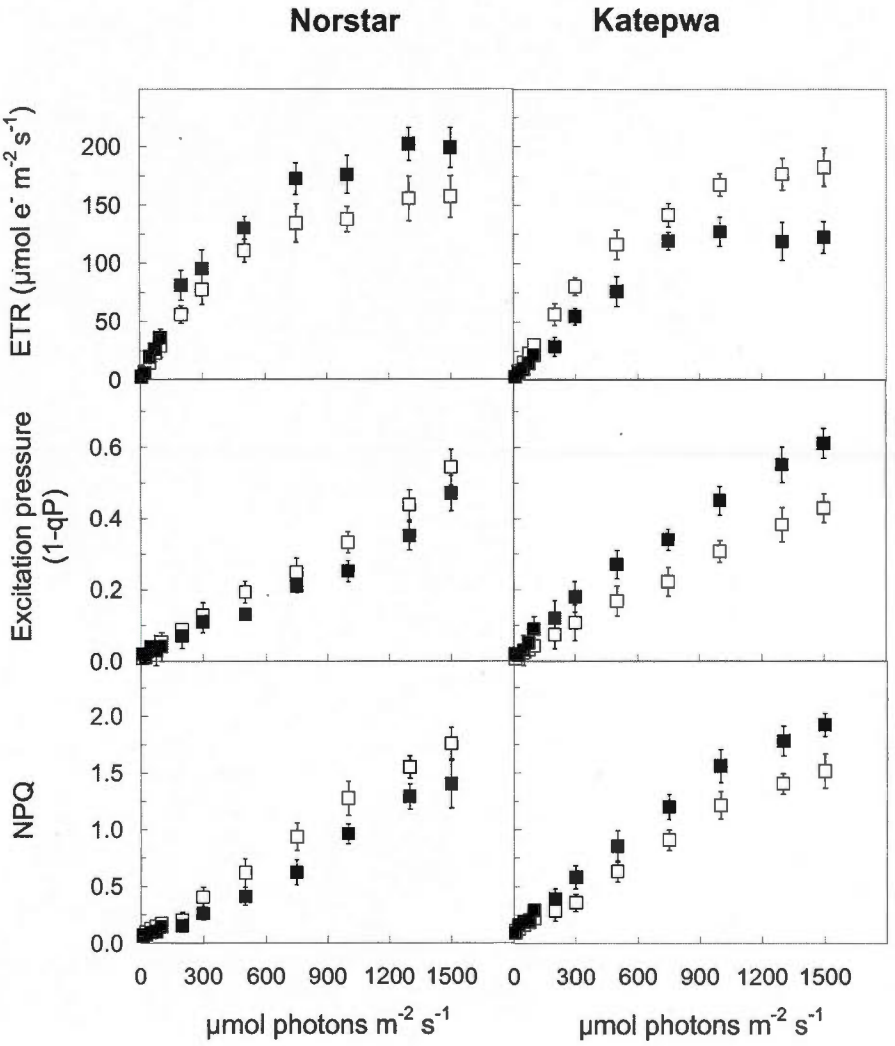

Figure 2.S7: Light response curves of electron transport rates (ETR; A, B), excitation pressures (1-qP; C, D) and non- photochemical quenching of excess energy (NPQ; E, F) for Norstar winter (A, C, E) and Katepwa spring wheat (B, D, F) grown at either 20°C (□) or 5°C (■) and at ambient CO2. Measurements were carried out at 20°C for both NA and CA plants. Each point represents the mean of nine plants from three different pots. Bars represent SD.

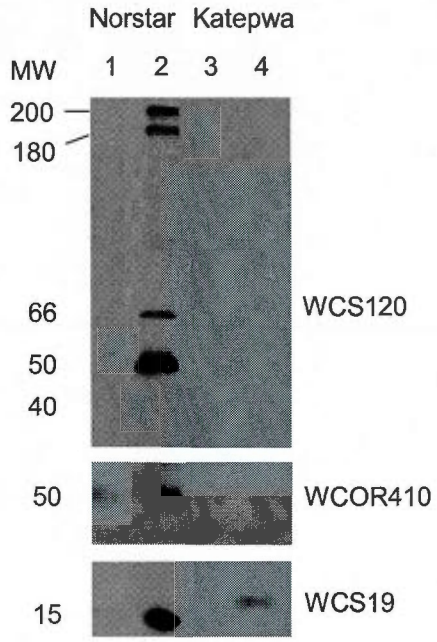

Figure 2.S8: Immunoblot analyses of WCS120, WCOR410 and WCS19 in winter (cv Norstar) and spring (cv Katepwa) wheat grown at either 20°C (NA) or 5°C (CA). The polypeptides were identified by anti - WCS120 (1/20000), - WCOR410 (1/10000), and – WCS19 (1/5000) antibodies. Molecular weight (MW) is indicated in kDa on the left. Lane 1 = NA Norstar, Lane 2 = CA Norstar, Lane 3 = NA Katepwa, Lane 4 = CA Katepwa.

2.9 REFERENCES

Adams III WW, Demmig-Adams B, Rosenstiel TN, Brightwell AK, Ebbert V (2002) Photosynthesis and photoprotection in overwintering plants. Plant Biol 4: 545-557

Amasino R (2004) Vernalization, Competence, and the Epigenetic Memory of Winter. Plant Cell 16: 2553-2559

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidases in *Beta vulgaris*. Plant Physiol 24: 1-15

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008) Structure and functional analysis of wheat ICE (Inducer of CBF Expression) genes. Plant Cell Physiol 49: 1237-1249

Boese SR, Hüner NPA (1990) Effect of growth temperature and temperature shifts on spinach leaf morphology and photosynthesis. Plant Physiol 94: 1830-1836

Boese SR, Hüner NPA (1992) Developmental history affects the susceptibility of spinach leaves to in vivo low temperature photoinhibition. Plant Physiol 99: 1141-1145

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci 12: 1360-1385

Coplen TB (1994) Reporting of stable hydrogen, carbon, and oxygen isotopic abundances. Pure & Appl Chem 66: 273-276

Doherty CJ, Buskirk H, Myers SJ, Thomashow MF (2009) Roles for *Arabidopsis* CAMTA transcription factors in cold–regulated gene expression and freezing tolerance. Plant Cell 21: 972-984

Ensminger I, Busch F, Hüner NPA (2006) Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol Plant 126: 28-44

Gilmour SJ, Fowler SG, Thomashow MF (2000a) Arabidopsis transcriptional activators CBF1, CBF2 and CBF3 have matching functional activities. Plant Mol Biol 54: 767-781

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000b) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124: 1854-1865

Gorsuch PA, Pandey S, Atkin OK (2010a) Thermal de-acclimation: how permanent are leaf phenotypes when cold-acclimated plants experience warming? Plant Cell Environ 33: 1124-1137

Gorsuch PA, Pandey S, Atkin OK (2010b) Temporal heterogeneity of cold acclimation phenotypes in Arabidopsis leaves. Plant Cell Environ 33: 244-258

Gray GR, Chauvin LP, Sarhan F, Hüner NPA (1997) Cold acclimation and freezing tolerance. A complex interaction of light and temperature. Plant Physiol 114: 467-474

Gray GR, Savitch LV, Ivanov A, Hüner NPA (1996) Photosystem II excitation pressure and development of resistance to photoinhibition II. Adjustment of photosynthetic capacity in winter wheat and winter rye. Plant Physiol 110: 61-71

Guy CL (1990) Cold acclimation and freezing tolerance: role of protein metabolism. Ann Rev Plant Physiol Plant Mol Biol 41: 187-223

Guy CL, Huber JLA, Huber SC (1992) Sucrose phosphate synthase and sucrose accumulation at low temperature. Plant Physiol 100: 502-508

Hüner NPA (1985) Morphological, anatomical and molecular consequences of growth and development at low temperature in *Secale cereale* L. cv Puma. Amer J Bot 72: 1290-1306

Hüner NPA, Elfman B, Krol M, MacIntosh A (1984) Growth and development at cold hardening temperatures. Chloroplast ultrastructure, pigment content and composition. Can J Bot 62: 53-60

Hüner NPA, Öquist G, Hurry VM, Krol M, Falk S, Griffith M (1993) Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. Photosyn Res 37: 19-39

Hüner NPA, Öquist G, Sarhan F (1998) Energy balance and acclimation to light and cold. Trend Plant Sci 3: 224-230

Hüner NPA, Palta JP, Li PH, Carter JV (1981) Anatomical changes in leaves of Puma rye in response to growth at cold hardening temperatures. Bot Gaz 142: 55-62

Hurry VM, Hüner NPA (1991) Low growth temperature effects a differential inhibition of photosynthesis in spring and winter wheat. Plant Physiol 96: 491-497

Hurry VM, Hüner NPA (1992) Effects of cold hardening on sensitivity of winter and spring wheat leaves to short-term photoinhibition and recovery of photosynthesis. Plant Physiol 100: 1283-1290

Hurry V, Hüner NPA, Selstam E, Gardestrom P, Öquist G (1996) Photosynthesis at low temperatures. In: Raghavendra AS (ed) Photosynthesis: a comprehensive treatise. Cambridge Univ Press, pp 238-249

Hurry VM, Krol M, Öquist G, Hüner NPA (1992) Effect of long-term photoinhibition on growth and photosynthesis of cold hardened spring and winter wheat. Planta 188: 369-375

Hurry VM, Malmberg G, Gardeström P, Öquist G (1994) Effects of a short-term shift to low temperature and of long-term cold hardening on photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase and sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). Plant Physiol 106: 983-990

Hurry V, Strand A, Furbank R, Stitt M (2000) The role of inorganic phosphate in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature is revealed by the pho mutants of *Arabidopsis thaliana*. Plant J 24: 383-96

Hurry VM, Strand A, Tabiaeson M, Gardeström P, Öquist G (1995) Cold hardening of spring and winter wheat and rape results in differential effects on growth, carbon metabolism, and carbohydrate content. Plant Physiol 109: 697-706

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces COR genes and enhances freezing tolerance. Science 280: 104-106

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotech 17: 287-291

Krause GH (1988) Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. Physiol Plant 74: 566-74

Lapointe L, Hüner NPA (1993) Photoinhibition of intact isolated mesophyll cells from rye. Plant Cell Environ 16: 249-58

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10: 1391-1406

McDonald A, Ivanov AG, Bode R, Maxwell D, Rodermel SR, Hüner NPA (2011) Flexibility in photosynthetic electron transport: the physiological role of plastoquinol terminal oxidase (PTOX). Biochim Biophys Acta (Bioenergetics), in press.

O'Leary MH (1988) Carbon isotopes in photosynthesis: Fractionation techniques may reveal new aspects of carbon dynamics in plants. Bio Sci 38: 328-336

Öquist G, Hüner NPA (2003) Photosynthesis of overwintering evergreen plants. Ann Rev Plant Biol 54: 329-355

Öquist G, Hurry VM, Hüner NPA (1993) Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. Plant Physiol 101: 245-250

Pocock TH, Hurry VM, Savitch LV, Hüner NPA (2001) Susceptibility to low-temperature photoinhibition and the acquisition of freezing tolerance in winter and spring wheat: The role of growth temperature and irradiance. Physiol Plant 113: 499-506

Rapacz M, Wolanin B, Hura K, Tyrka M (2008) The effects of cold acclimation on photosynthetic apparatus and the expression of COR14b in four genotypes of barley (*Hordeum vulgare*) contrasting in their tolerance to freezing and high-light treatment in cold conditions. Ann Bot 101: 689–699

Rosso D, Bode R, Li W, Krol M, Saccon D, Wang S, Schillaci LA, Rodermel SR, Maxwell DP, Hüner NPA (2009) Photosynthetic redox imbalance governs leaf

sectoring in the Arabidopsis thaliana variegation mutants immutans, spotty, *var1*, and *var2* . Plant Cell 21: 3473–3492

Sarhan F, Ouellet F, Vazquez-Tello A (1997) The wheat *Wcs120* gene family: a useful model to understand the molecular genetics of freezing tolerance in cereals. Physiol Plant 101: 439-445

Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Hüner NPA, Shinozaki K, Singh J (2005) The effect of overexpression of two Brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. Plant Cell Physiol 46: 1525-1539

Savitch LV, Barker-Astrom J, Ivanov A G, Hurry V, Oquist G, Hüner NPA (2001) Cold acclimation of *Arabidopsis thaliana* results in incomplete recovery of photosynthetic capacity which is associated with an increased reduction of the chloroplast stroma. Planta 214: 295-301

Savitch LV, Harney T, Hüner NPA (2000) Sucrose metabolism in spring and winter wheat in response to high irradiance, cold stress and cold acclimation. Physiol Plant 108: 270-278

Savitch LV, Leonardos ED, Krol M, Jansson S, Grodzinski B, Hüner NPA, Öquist G (2002) Two different strategies for light utilization in photosynthesis in relation to growth and cold acclimation. Plant Cell Environ 25: 761-771

Somersalo S, Krause GH (1989) Photoinhibition at chilling temperatures : Fluorescence characteristics of unhardened and cold-hardened spinach leaves. Planta 177: 409-416

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Ann Rev Plant Physiol 35: 543-584

Stitt M, Hurry VM (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Curr Opin Plant Biol 5: 199-206

Strand A, Foyer CH, Gustafsson P, Gardestrom P, Hurry V (2003) Altering flux through the sucrose biosynthesis pathway in transgenic Arabidopsis thaliana modifies photosynthetic acclimation at low temperatures and the development of freezing tolerance. Plant Cell Environ 26: 523-535

Strand A, Hurry V, Gustafsson P, Gardestrom P (1997) Development of *Arabidopsis thaliana* leaves at low temperature releases the suppression of photosynthesis

and photosynthetic expression despite the accumulation of soluble carbohydrates. Plant J 12: 605-614

Strand A, Hurry VM, Henkes S, Hüner NPA, Gustafsson P, Gardeström P, Stitt M (1999) Acclimation of Arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol 119: 1387-1398

Sung S, Amasino RM (2005) Remembering winter: Toward a molecular understanding of vernalization. Ann Rev Plant Biol 56: 491-508

Thomashow MF (2001) So what's new in the field of plant cold acclimation? Lots! Plant Physiol 125: 89-93

Van Buskirk HA, Thomashow MF (2006) Arabidopsis transcription factors regulating cold acclimation. Physiol Plant 126: 72-80

Wilson K E, Ivanov A G, Oquist G, Grodzinski B, Sarhan F, Hüner NPA (2006) Energy balance, organellar redox status and acclimation to environmental stress. Can J Bot 84: 1355-1370.

CHAPITRE III

ARTICLE II: COLD ACCLIMATION INHIBITS CO2-DEPENDENT STIMULATION OF PHOTOSYNTHESIS IN SPRING WHEAT AND SPRING RYE

Keshav Dahal, Khalil Kane, Fathey Sarhan, Bernard Grodzinski, Norman PA Hüner

Article publié dans la revue *Journal of Botany*. 2012b, volume 90(6) p. 433-444

Contribution

J'ai participé dans quelques étapes d'expérimentation et de rédaction de l'article sous la supervision et le soutien de mon directeur Fathey Sarhan et de mes collaborateurs, Keshav Dahal et des professeurs Norman Hüner et Bernard Grodzinski. J'ai effectué les expériences de PCR en temps réel et de western blot des protéines CORs régulés par le froid. J'ai été impliqué dans la rédaction et la correction du manuscrit avant la soumission.

3.1 RÉSUMÉ

Nous avons déterminé si l'exposition à court terme aux concentrations élevées de CO_2 de 700 µmol C mol^{-1} peut compenser l'inhibition photosynthétique induite par le froid chez les variétés printanières. Les taux d'assimilation de la photosynthèse (Asat) ont été mesurées chez deux variétés (hivernale et printanière) de blé *Triticum aestivum* (cv Norstar et Katepwa) et de seigle *Secale cereale* (cv Musketeer et SR4A) non acclimatées (température 20/16 °C jour/nuit) et acclimatées au froid (Température 5/5 °C jour/nuit). Suite à une exposition courte de 80h aux concentrations élevées CO_2 de 700 µmol C mol^{-1}, les variétés printanières de blé et seigle acclimatées au froid présentent une diminution de 45 à 60% de leur taux de photosynthèse comparé à leurs homologues non acclimatées. Les plants de blé et le seigle d'hiver acclimatés au froid présentent une augmentation de 15 à 35% de leur photosynthèse comparativement aux plants non acclimatés. L'assimilation de CO_2 durant l'acclimatation au froid est 60% moins élevé chez les variétés printanières comparées à celles hivernales. Ces résultats démontrent que l'exposition à court terme aux concentrations élevées de CO_2 ne peut compenser l'inhibition photosynthétique induite par le froid chez les variétés printanières. La limitation du CO_2 pour la Ribulose 1, 5-biphosphate carboxylase/oxygénase que l'on observe généralement à concentration ambiante de CO_2 est accentuée par l'acclimatation au froid chez les cultivars de printemps. De plus, l'exposition à court terme aux concentrations élevées de CO_2, ne permet pas aux cultivars de printemps d'ajuster la sensibilité thermale associée à la photosynthèse durant l'acclimatation au froid comparativement aux cultivars d'hiver.

3.2 ABSTRACT

We assessed the effects of short-term elevated CO_2 on the light- saturated rates of photosynthesis (A_{sat}) of spring (cv SR4A, cv Katepwa) and winter (cv Musketeer, cv Norstar) wheat (*Triticum aestivum*) and rye (*Secale cereale*) cultivars grown at ambient CO_2 (380 μmol C mol^{-1}) at either 20/16°C (non-acclimated, NA) or 5/5°C (cold acclimated, CA). In spring wheat/rye, cold acclimation decreased CO_2-stimulation of A_{sat} by 45 - 60% relative to NA controls following a short-term (80 h) shift of plants from ambient to elevated CO_2 (700 μmol C mol^{-1}). In contrast, in winter wheat/rye, cold acclimation enhanced CO_2-stimulation of A_{sat} by 15-35% relative to NA controls upon a shift to elevated CO_2. The stimulation observed for CA spring cultivars was about 60% less than that of CA winter cultivars. We conclude that a short-term exposure of spring cultivars to elevated CO_2 cannot compensate for the cold acclimation-induced inhibition of A_{sat}. Cold acclimation of spring cultivars appeared to exacerbate Rubisco CO_2 substrate limitations present under ambient CO_2. Furthermore, CA spring cultivars were unable to adjust their short-term temperature sensitivity of A_{sat} under elevated CO_2 compared to the winter cultivars.

3.3 INTRODUCTION

Acclimation of the photosynthetic apparatus to low, non-freezing temperatures has been extensively studied in both cold-sensitive and cold-tolerant species (Adams *et al.,* 2002; Ensminger *et al.,* 2006). It is well documented that low growth temperature is often stressful to many cold-sensitive cultivars and species such as spring wheat (Hurry and Hüner 1991), spring rape (Hurry *et al.,* 1995) and tomato (Sassernath and Ort 1990). Spring cereals exhibit decreased photosynthetic capacity estimated as light-saturated rates of carbon assimilation, A_{sat}, in response to growth and development at low temperatures (Hurry and Hüner 1991). Spring cultivars exhibit a limited sink demand and subsequent retardation of carbon export to the sinks during cold acclimation (Hurry *et al.,* 1995). This results in inhibition of sucrose synthesis and concomitant decrease in utilization of phosphorylated intermediates and depletion in stromal P_i which leads to feedback-limited photosynthesis (Hurry et. al. 1995; Savitch *et al.,* 2002). Therefore, spring cultivars appear to exhibit decreased plasticity to low growth temperatures and exhibit reduced photosynthetic capacity during cold acclimation (Hurry *et al.,* 1995; Savitch *et al.,* 2002). In addition, spring cultivars exhibit susceptibility to low temperature-induced photoinhibition of photosynthesis during the cold acclimation process (Hüner *et al.,* 1993; Hurry *et al.,* 1995; Pocock *et al.,* 2001) and are unable to attain maximum freezing tolerance in winter (Sarhan *et al.,* 1997).

In contrast, cold-tolerant cultivars and species such as winter wheat, winter rye, winter barley, winter rape, spinach and *Arabidopsis thaliana* exhibit enhanced photosynthetic capacity during the cold acclimation process (Hurry and Hüner 1991; Öquist *et al.,* 1993; Hurry *et al.,* 1994, 1995; Hüner *et al.,* 1998; Savitch *et al.,* 2002; Rapacz *et al.,* 2008). It has been suggested that the increase in photosynthetic capacity upon cold acclimation of cold-tolerant cultivars and species is, in part, the result of the enhanced activities of key regulatory photosynthetic enzymes such as

Rubisco, cFBPase, and SPS (Hurry *et al.,* 1994, 1995; Stitt and Hurry 2002) and concomitant carbon export to the sinks during cold acclimation (Leonardos *et al.,* 2003). These cultivars and species require growth and development at low temperatures and enhanced carbon assimilation in order to attain maximum freezing tolerance (Öquist & Hüner 2003) as well as to develop increased resistance to low temperature-induced photoinhibition (Krause 1988; Somersalo and Krause 1989; Boese and Hüner 1992; Hüner *et al.,* 1993; Öquist *et al.,* 1993; Pocock *et al.,* 2001; Savitch *et al.,* 2001).

It has been well documented that short–term exposure of C_3 plants from ambient to elevated CO_2 results in an immediate increase in the rates of net CO_2 assimilation (Cheng *et al.,* 1998; Long *et al.,* 2004; Ainsworth and Rogers 2007). This stimulation of photosynthesis in C_3 plants due to elevated CO_2 is thought to occur for the following reasons. First, the K_m (CO_2) for Rubisco is close to the current atmospheric CO_2 (Long *et al.,* 2004, Tcherkez *et al.,* 2006) which indicates that Rubisco is CO_2 substrate-limited at ambient CO_2. Thus, an immediate increase in carboxylation velocity is expected by increased CO_2 substrate availability. Second, elevated CO_2 competitively inhibits the light-dependent evolution of CO_2 by photorespiration because CO_2 is a competitive inhibitor of the oxygenation of RuBP by Rubisco (Long *et al.,* 2004).

Much of our present knowledge of photosynthetic acclimation of winter and spring cereals to low temperature has been achieved through comparative studies of plants grown and developed at ambient CO_2. Previous studies have suggested that cold acclimation of winter cereals enhance the light-saturated rates of CO_2 assimilation measured on a leaf area basis primarily due to increased specific leaf weight (Hurry and Hüner 1991; Hurry *et al.,* 1995). In contrast, spring cereals exhibit a decreased A_{sat} in response to low growth temperature. Does a short-term exposure

of spring wheat and spring rye to elevated CO_2 compensate for the cold acclimation-induced inhibition of CO_2 assimilation?

To address this question, we compared CO_2 gas exchange rates as well as photosynthetic electron transport coupled with photosynthetic gene expression and polypeptide accumulation in NA and CA spring cultivars of wheat and rye grown at ambient CO_2 (380 μmol C mol^{-1}) and subsequently exposed to short-term (80 h) elevated CO_2 (700 μmol C mol^{-1}). As an additional control, NA and CA winter wheat and winter rye were also exposed to short-term elevated CO_2 for comparison.

3.4 RESULTS

3.4.1 Effects of cold acclimation on growth characteristics at ambient CO_2

Cold acclimation reduced growth rates by about 70% relative to the NA counterparts regardless of species or cultivars (Table1). The third leaves of all NA cultivars were fully expanded between 20-25 days and those of CA cultivars between 70-80 days. Consequently, all subsequent comparisons were made on fully expanded third leaves of 25-day-old NA and 75-day-old CA wheat and rye cultivars.

CA winter cultivars exhibited a 1.9 to 2.3-fold increase in the specific leaf weight (SLW, g dry weight m^{-2} leaf area) relative to those values observed for NA counterparts whereas the SLW changed minimally in CA versus NA spring cultivars (Table 1). These results are consistent with published data on the differential effects of cold acclimation on SLW for winter and spring cereals (Hüner *et al.,* 1981, 1985; Gray *et al.,* 1996; Leonardos *et al.,* 2003). Pot size had minimal effects on cold acclimation-induced changes on plant morphology and total plant dry matter accumulation in all cultivars tested (Table S2).

3.4.2 Effects of cold acclimation and measuring temperatures on light-saturated rates of photosynthesis, Asat, and stomatal conductance at ambient CO2

We observed comparable gross A_{sat} of 18 - 22 μmol CO_2 m^{-2} s^{-1} for all NA winter and spring cultivars grown and measured at ambient CO_2 at 20°C (Fig. 1A, white bars, 20). A decrease in the measuring temperature from 20° to 5°C inhibited these rates by about 35 - 50% in all NA cultivars (Fig. 1A, white bars, 5). However, the CA winter cultivars, Musketeer rye and Norstar wheat, were able to maintain gross CO_2 assimilation rates at 5°C (Fig. 1A, black bars, 5) that were comparable to or slightly higher than those rates observed for NA controls measured at 20°C (Fig.

1A, white bars, 20). The measuring temperature had minimal effects on gross A_{sat} of CA Musketeer and Norstar (Fig. 1A, black bars, 20 versus 5). In addition, gross A_{sat} measured at 5°C was 70-130% higher in CA Musketeer and Norstar (Fig. 1A, black bars, 5) as compared to those rates observed for their NA controls measured at the same temperature (Fig. 1A, white bars, 5). Thus, cold acclimation of Musketeer winter rye and Norstar winter wheat enhanced the light-saturated rates of gross CO_2 assimilation irrespective of the measuring temperature.

In contrast to the CA winter cultivars, the CA spring cultivars, SR4A rye and Katepwa wheat, exhibited a 30 - 45% decrease in the light-saturated rates of gross CO_2 assimilation regardless of measuring temperature (Fig. 1A, black bars, 5, 20) relative to the NA controls measured at 20°C (Fig. 1, white bars, 20). The assimilation rates observed for CA spring cultivars, SR4A and Katepwa were comparable to those rates observed for NA controls but measured at 5°C (Fig. 1A, white versus black bars, 5). Hence, the CA-induced decrease in the light-saturated rates of gross CO_2 assimilation appeared to be specific for the spring cultivars tested. However, irrespective of species or cultivar, growth at low temperature appeared to decrease the temperature sensitivity of CO_2 assimilation. A change of measuring temperature affected A_{sat} by only 5-10% in all CA cultivars whereas it affected all NA cultivars by 35-60% (Fig.1A).

Cold acclimation suppressed the stomatal conductance by 30 - 60% when measured at 20°C (Fig 1B, 20, black bars versus white bars) and by 15-25% when measured at 5°C in all four cultivars (Fig 1B, 5, black bars versus white bars). Thus, decrease in stomatal conductance is not due to the fact that CA plants are at lower temperature than NA plants because stomatal conductance was measured at 5°C and at 20°C for both CA and NA plants (Fig. 1B). The decrease in stomatal conductance was evident regardless of the leaf measuring temperature. Furthermore, stomatal

conductance of the CA winter cultivars appeared to be less temperature sensitive than their NA counterparts whereas CA and NA spring cultivars appeared to be equally sensitive to measuring temperatures (Fig 1B).

3.4.3 Effects of short-term elevated CO2 on light response curves and light-saturated CO2 response curves

Fig. 2 illustrates the effects of short-term elevated CO_2 on light response curves for Musketeer winter (Fig. 2a, b) and SR4A spring rye (Fig. 2c, d). Exposure to elevated CO_2 significantly increased the apparent maximum quantum efficiency by about 25-45% compared to that of at ambient CO_2 in NA Musketeer (Fig. 2a, closed versus open circles, Table 2, Q) and NA SR4A rye (Fig. 2c, closed versus open circles, Table 2, Q). Whereas elevated CO_2 had minimal effects on Q values for CA Musketeer (Fig. 2b, closed versus open circles, Table 2, Q) as well as for CA SR4A (Fig. 2d, closed versus open circles, Table 2, Q). Exposure to elevated CO_2 enhanced the light-saturated rates of gross photosynthesis (gross A_{sat}) by 55 - 65% in NA Musketeer and SR4A rye (Fig. 2a, c, closed versus open circles). In contrast, the gross A_{sat} increased by 70% in CA Musketeer but increased by minimally in CA SR4A rye following exposure to elevated CO_2 (Fig. 2b, d, closed versus open circles). Comparable results were obtained for light response curves of Norstar winter and Katepwa spring wheat (Fig. S1).

Exposure to elevated CO_2 had minimal effects on the CO_2 limited-carboxylation efficiency as well as the light-saturated and CO_2-saturated carboxylation capacity relative to those values observed at ambient CO_2 for all cultivars irrespective of acclimation state (Fig. 3, Fig. S2, Table 2, CE). Similarly there were no CO_2 effects on the maximum Rubisco carboxylation capacity (V_{cmax}) as well as maximum rates of electron transport (J_{max}) for all cultivars regardless of acclimation state (Table 2).

3.4.4 Kinetics for the CO2 stimulation of the light-saturated rates of gross photosynthesis

All NA cultivars exhibited average gross A_{sat} of 18 - 22 μmol CO_2 m^{-2} s^{-1} when grown and measured at ambient CO_2 and 20°C (Fig. 4a,c, Fig. S3a, c, open circles). A shift from ambient (Fig. 4a, open circles) to elevated CO_2 (Fig. 4a, closed circles) at 20°C stimulated average gross CO_2 assimilation by 63% in NA Musketeer winter rye. This CO_2-stimulation of A_{sat} was observed within 30 min of shift from ambient to elevated CO_2 and was fairly stable throughout the entire shift experiment. Comparable CO_2-stimulation of gross A_{sat} (54%) was observed for NA SR4A spring rye (Fig. 4c, open versus closed circles) as well as for NA Norstar winter wheat (49%, Fig. S3a, open versus closed circles) and NA Katepwa spring wheat (58%, Fig. S3c, open versus closed circles). Thus, NA winter and spring cultivars exhibited a comparable stimulation of A_{sat} upon a short-term exposure from ambient to elevated CO_2.

CA Musketeer winter rye exhibited an average 70% stimulation of light-saturated rates of gross CO_2 assimilation following exposure to elevated CO_2 at 5°C (Fig. 4b, closed circles) relative to those rates observed at ambient CO_2 at 5°C (Fig. 4b, open circles). Even when measured at its growth temperature of 5°C, the average CO_2-stimulated rate of gross CO_2 assimilation was 37% higher for CA Musketeer (Fig. 4b, closed circles, 42.8 ± 3.2 μmol CO_2 m^{-2} s^{-1}) as compared to the stimulation observed for NA Musketeer but measured at 20°C (Fig 4a, closed circles, 31.3 ± 2.4 μmol CO_2 m^{-2} s^{-1}). Similar trends were observed for Norstar winter wheat (Fig. S3a versus S3b). Thus, cold acclimation further enhanced the CO_2- stimulation of gross A_{sat} in winter cultivars, Musketeer and Norstar.

In contrast to CA Musketeer winter rye, CA spring rye (SR4A) exhibited minimal stimulation of gross CO_2 assimilation following shift to elevated CO_2 at 5°C

(Fig. 4d, closed circles) relative to those rates observed at ambient CO_2 at 5°C (Fig. 4d, open circles). Hence, since cold acclimation considerably inhibited A_{sat} relative to that of NA spring rye, this minimal stimulation of A_{sat} of CA spring rye at elevated CO_2 (Fig. 4d, closed circles) was not sufficient to compensate for the cold acclimation-induced inhibition at ambient CO_2 (Fig. 4c versus 4d, open circles). The average CO_2-stimulated rate of gross CO_2 assimilation measured at 5°C was 49% less for CA spring rye (Fig. 4d, closed circles, 17.5 ± 3.2 μmol CO_2 m^{-2} s^{-1}) as compared to the stimulation observed for NA spring rye measured at 20°C (Fig 4c, closed circles, 34.3 ± 2.4 μmol CO_2 m^{-2} s^{-1}). In addition, the stimulation observed for CA spring rye was 59% less than that of CA winter rye measured at 5°C (Fig 4b versus 4d, closed circles). We observed similar trends for CA Katepwa spring wheat (Fig. S3c versus S3d) relative to CA Norstar winter wheat. For instance, cold acclimation inhibited CO_2-dependent stimulation of photosynthesis by 58% in Katepwa spring wheat (Fig. S3c versus S3d, closed circles) whereas cold acclimation further enhanced the CO_2-dependent stimulation of photosynthesis by 12% in Norstar winter wheat (Fig. S3a versus S3b, closed circles). Thus, cold acclimation inhibited the CO_2- stimulation of gross A_{sat} in spring cultivars, SR4A and Katepwa.

3.4.5 Effects of short-term elevated CO2 on stomatal conductance

Exposure to elevated CO_2 suppressed leaf stomatal conductance by about 40% in all NA cultivars and, as a consequence, leaf transpiration rates decreased by 25 - 40% (Table 3). In contrast, except for CA Katepwa spring wheat, elevated CO_2 induced minimal changes in stomatal conductance as well as leaf transpiration rates in all other CA cultivars (Table 3). Therefore, the enhanced photosynthetic capacity of all cultivars associated with short-term exposure to elevated CO_2 can not be due to increased stomatal conductance. Leaf water use efficiency (WUE) of all NA cultivars increased by 160 -180% following exposure to elevated CO_2 whereas leaf WUE of all CA cultivars increased by about 50 - 75% upon exposure to elevated CO_2 (Table

3). However, WUE was about 50% lower in CA spring cultivars than in CA winter cultivars irrespective of CO_2 concentrations.

3.4.6 Temperature sensitivity of CO2-stimulated Asat

Since we observed maximum CO_2 stimulation within an hour of shift from ambient to elevated CO_2, we measured the temperature sensitivity of the 1 h temperature shift on the CO_2-dependent stimulation of light-saturated photosynthetic rates for Musketeer winter and SR4A spring rye (Fig. 5). Results in Fig. 5 illustrate the temperature response curves for CO_2 assimilation in Musketeer (Fig. 5a, b) and SR4A rye (Fig. 5c, d) following a 1 h shift to the various temperatures indicated at either ambient (open circles) or elevated CO_2 (closed circles). In the NA state, the temperature sensitivity for net A_{sat} were similar for both cultivars with a maximum A_{sat} at 25°C at either ambient or elevated CO_2 (Fig. 5a, c). However, A_{sat} in Musketeer and SR4A appeared to be less sensitive to temperatures between 5° - 25°C in the CA than in the NA state irrespective of CO_2 concentration (Fig. 5b, d). As a consequence, the extent of the elevated CO_2-induced stimulation of A_{sat} after 1 h at the respective temperatures was constant between 5° - 25°C in CA Musketeer and CA SR4A whereas the elevated CO_2-induced stimulation of A_{sat} was at a minimum at 5°C and increased to a maximum at 25°C in NA counterparts. At temperatures above 25°C, A_{sat} decreased substantially in NA Musketeer and SR4A at ambient CO_2 (Fig. 5a, c, open circles) but changed minimally at elevated CO_2 (Fig. 5a, c, closed circles). Similar results were observed for CA Musketeer (Fig. 5b). In contrast, A_{sat} in CA SR4A decreased to a comparable extent at either ambient or elevated CO_2 at temperatures above 25°C (Fig. 5d). Similar trends for the sensitivity of a 1h temperature shift on the CO_2-dependent stimulation of light-saturated photosynthetic rates were observed for winter and spring wheat (Fig. S4).

In order to assess the sensitivity of a prolonged shift to high temperature on the CO_2-dependent stimulation of light-saturated photosynthetic rates, we measured the CO_2 assimilation rates for Musketeer winter and SR4A spring rye after of shift to various temperatures at either ambient or elevated CO_2 (Fig. 6). Consistent with the 1 h temperature shift, similar results were observed for an temperature shift except that, at temperatures above 25°C, A_{sat} decreased substantially not only at ambient CO_2 (Fig. 6a, b, c, d, open circles) but also at elevated CO_2 (Fig. 6a, b, c, d, closed circles) in both winter and spring rye regardless of acclimation state. Comparable trends were observed for winter and spring wheat (Fig. S5a, b, c, d).

To illustrate the relative effects of elevated CO_2 on temperature-dependent inhibition of net A_{sat}, we calculated the difference in net A_{sat} between elevated CO_2 (ECO_2) versus ambient CO_2 (ACO_2) after 1 h of shift to various temperatures (Fig. 5e, f). In the NA state, the temperature profile of ECO_2 - ACO_2 for Musketeer (Fig. 5e, open triangles) was indistinguishable from that of SR4A (Fig. 5e, closed triangles) and increased linearly with increased temperatures between 5° - 40°C. In contrast, ECO_2-ACO_2 for CA spring rye remained constant but low at all temperatures between 5° - 40°C (Fig. 5f, closed triangles), whereas ECO_2 - ACO_2 for CA Musketeer (Fig. 5f, open triangles) was at least 3-fold higher between 5° and 25°C and increased between 25° and 40°C relative to that of SR4A.

Similar trends were observed for a temperature shift except that, at temperatures above 25°C, ECO_2 - ACO_2 decreased substantially in both cultivars regardless of acclimation state and CO_2 concentrations (Fig. 6e, f). Comparable results were observed for winter and spring wheat (Fig. S4e, f, fig. S5e, f). Thus, the CO_2-dependent stimulation of CO_2 assimilation appeared to be less sensitive to increased temperatures in all CA cultivars relative to their NA counterparts.

3.4.7 Effects of short- term elevated CO2 on dark respiratory rates

Unlike photosynthesis, 80h exposure to elevated CO_2 at their respective growth temperature minimally (5-20%) decreased dark respiratory rates in all NA and CA cultivars (Table S3). However, an exposure to 40°C at either ambient (380 μmol C mol^{-1}) or elevated (700 μmol C mol^{-1}) CO_2 substantially suppressed respiratory rates (40-70%) in all cultivars irrespective of acclimation state (Table S3).

3.4.8 Effects of short-term elevated CO2 and high temperature on leaf protein content, photosynthetic gene expression and polypeptide content

We observed a comparable leaf protein content of 2.3 - 3.3 g m^{-2} leaf area for all NA cultivars at ambient CO_2 (Table 4). CA winter wheat and winter rye exhibited a 4.2 to 4.7–fold increase in leaf protein content compared to NA controls (Table 4). In contrast, CA spring wheat and spring rye exhibited minimal changes in leaf protein content relative to their NA counterparts (Table 4). Exposure to elevated CO_2 for , at their respective growth temperature, induced minimal changes on leaf protein content in all four cultivars regardless of acclimation state (Table 4). However, shifting plants to 40°C for at either ambient or elevated CO_2 decreased the leaf protein content by 40 - 60% in all NA and CA cultivars (Table 4).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) indicated that exposure to 40°C at ambient CO_2 considerably reduced the transcript levels of most of the key chloroplast (rbcL, psbA, psaA) and nuclear encoded (cFBPase, Lhcb1) photosynthetic genes examined in both Norstar and Katepwa wheat (Fig. 7). However, Lhcb1 expression in Katepwa was the least sensitive to high temperature (Fig.7). This general reduction of transcript levels occurred independently of acclimation state. Similar results were obtained for plants exposed to 40°C at elevated CO_2 (Fig.7).

Cold acclimation induced minimal changes (10 - 15%) in the protein levels of rbcL, cFBPase, Lhcb1, PsbA and PsaA in either winter or spring cultivars at ambient CO_2 (Fig. 8). This can be explained by the fact that the loading for SDS-PAGE and immunoblotting was based on leaf protein. Our leaf protein results (Table 4) have indicated that CA winter cultivars exhibited a 4.5-fold increase in leaf protein content per unit leaf area relative to NA controls. Hence, the increased leaf protein content is likely to reflect the enhanced levels of rbcL, cFBPase, Lhcb1, PsbA and PsaA on a leaf area basis for CA winter cultivars, Norstar and Musketeer. However, the proportion of the photosynthetic polypeptides tested relative to total leaf protein does not change upon cold acclimation.

In contrast, CA spring cultivars, Katepwa wheat and SR4A rye exhibited minimal changes in leaf protein content (Table 4) and would likely reflect minimal changes in the levels of all five proteins tested on a leaf area basis.

Exposure to 40°C at ambient CO_2 for substantially reduced the abundance of photosynthetic enzymes, cFBPase and thylakoid proteins, Lhcb1, psbA, psaA by 20 - 40% regardless of acclimation state and cultivars (Fig. 8). However, rbcL content appeared to be least sensitive to high temperature (Fig. 8). Comparable results were observed for plants exposed to 40°C at elevated CO_2 (Fig. 8).

3.5 DISCUSSION

Since spring cereals exhibit a decreased A_{sat} in response to low growth temperature at ambient CO_2, we asked the question whether a short-term exposure of spring wheat and spring rye from ambient to elevated CO_2 could compensate for the cold acclimation-induced inhibition of CO_2 assimilation. Our results have clearly indicated that a short-term exposure of Katepwa spring wheat and SR4A spring rye to elevated CO_2 can not compensate for the cold acclimation-induced inhibition of CO_2 assimilation. The cold acclimation-induced decrease in A_{sat} of spring cultivars, SR4A and Katepwa, at ambient CO_2 resulted in a 45% (SR4A) and a 60% (Katepwa) inhibition of CO_2-dependent stimulation of photosynthesis during the short-term shift to elevated CO_2 relative to NA controls. Therefore, even though spring wheat and rye grow at low temperature, cold acclimation not only inhibits photosynthetic rates at ambient CO_2 but also inhibits the stimulation of photosynthetic rates by short-term exposure to elevated CO_2. In addition, the CA spring wheat and spring rye are not able to recover from the low temperature-induced inhibition of A_{sat} through a short-term exposure to elevated CO_2. Cold acclimation-induced decrease in CO_2 assimilation of CA spring cultivars are consistent with the decrease in the maximum rates of photosynthetic electron transport, maximal quantum efficiency and maximal Rubisco carboxylation efficiency as well as carboxylation capacity in response to growth at low temperature (Table 2). The decreased A_{sat} in CA spring cultivars appears to be associated with a downregulation of carbon metabolism during cold acclimation (Hurry *et al.,* 1995). Previous studies have suggested that spring wheat and spring rape exhibit a limited sink demand and concomitant retardation of carbon export to the sinks during cold acclimation (Hurry *et al.,* 1995). As a consequence, the photosynthetic end-product, sucrose, accumulates in source leaves. This results in inhibition of sucrose synthesis and concomitant decrease in utilization of

phosphorylated intermediates and depletion in stromal P_i which leads to feedback-limited photosynthesis (Hurry et. al. 1995; Savitch *et al.,* 2002). In addition, the feedback inhibition of photosynthesis is also attributed to down-regulation of the expression and activities of key regulatory photosynthetic enzymes such as Rubisco, cFBPase, and SPS (Hurry *et al.,* 1995; Strand *et al.,* 1999). Therefore, spring cultivars appear to exhibit decreased plasticity to low growth temperatures and exhibit reduced photosynthetic capacity during cold acclimation (Hurry *et al.,* 1995; Savitch *et al.,* 2002). Consequently, spring cultivars, SR4A rye and Katepwa wheat were unable to compensate for the cold acclimation-induced inhibition of CO_2 assimilation following a short-term exposure to elevated CO_2.

In contrast to winter cultivars, the low temperature-induced increase in A_{sat} for winter cultivars, Musketeer and Norstar, at ambient CO_2, translated into 12% (Norstar) and 37% (Musketeer) increase in the capacity for carbon assimilation upon short-term exposure to elevated CO_2 relative to NA counterparts at elevated CO_2. This enhancement of photosynthetic capacity in CA versus NA winter cultivars can be explained by the fact that cold acclimation of the winter wheat and rye cultivars, results in major changes in leaf morphology and leaf anatomy (Hüner *et al.,* 1981; Hüner 1985; Gray *et al.,* 1996, Dahal *et al.,* 2011), which are associated with significant increases in SLW and the amount of photosynthetic apparatus per unit leaf area (Leonardos *et al.,* 2003, Dahal *et al.,* 2011). In addition, cold acclimation-induced increase in CO_2 assimilation of CA winter cultivars are consistent with the increase in the maximum rates of photosynthetic electron transport, maximal quantum efficieny and maximal Rubisco carboxylation efficiency as well as carboxylation capacity in response to growth at low temperature (Table 2).

CA spring cultivars appear to be incapable of adjusting the short-term temperature sensitivity of photosynthesis under elevated CO_2 compared to winter

cultivars. However, these differences are not reflected in a differential temperature stability of the photosynthetic gene expression either at the transcript level or at the level of photosynthetic polypeptide content associated with Rubisco, PSI and PSII. Thus, this differential temperature sensitivity between spring and winter cultivars is most likely due to differences in the relative temperature sensitivities of other components of both photorespiration and photosynthetic CO_2 assimilation.

It has been suggested that high temperature favors photorespiration compared to photosynthesis, due to differential aqueous solubilities of CO_2 and O_2 and kinetic properties of Rubisco (Long *et al.,* 2004; Salvucci and Crafts-Brandner 2004; Ainsworth and Rogers 2007). Thus, we suggest that the difference in the temperature profiles under ambient versus short-term elevated CO_2 are due to the differential effects of temperature on photosynthesis versus photorespiration. Under elevated CO_2, photorespiration is reduced and does not compete with photosynthesis whereas, under ambient CO_2, photorespiration competes with CO_2 assimilation.

We conclude that the inhibition of photosynthetic capacity after of exposure to 40°C at either ambient or elevated CO_2 (Fig. 6, fig. S5) is attributed to a general denaturation and degradation of leaf protein (Table 4) and concomitant decrease in the activities of major photosynthetic enzymes and components of photosynthetic electron transport such as rbcL, cFBPase, Lhcb1, psbA and psaA (Fig. 8). Furthermore, the inhibition of photosynthetic carbon assimilation is also closely correlated with a high temperature-induced decrease in the activation state of Rubisco as well as irreversible PSII damage (Salvucci and Crafts-Brandner 2004; Kumar *et al.,* 2009).

The K_m (CO_2) for Rubisco in air is 420 μmol mol^{-1} (Tcherkez *et al.*, 2006) which is close to the current ambient CO_2 of about 380 μmol C mol^{-1} (Long *et al.*, 2004). Thus, we suggest that the increased carbon assimilation upon the shift of plants from ambient to elevated CO_2 (700 μmol C mol^{-1}) resulted from increased Rubisco carboxylation activity as a consequence of increased CO_2 substrate availability. Our results clearly showed a marked suppression of stomatal conductance following exposure of NA plants to elevated CO_2 for all four cultivars. Therefore, we confirm that the increased photosynthetic capacity of all cultivars upon exposure to elevated CO_2 can not be accounted for by increased stomatal conductance but must reflect changes at the biochemical level. Since the maximum stimulation occurred in less than 30 min upon shift from ambient to elevated CO_2, we conclude that the short-term shift of plants to elevated CO_2 overcomes Rubisco CO_2 substrate limitations present under ambient CO_2. Cold acclimation of spring wheat and rye appeared to exacerbate this limitation.

In summary, cold acclimation of spring wheat and spring rye not only inhibits CO_2 assimilation under ambient CO_2 but also inhibits the capacity for the stimulation of light-saturated rates of photosynthesis during a short-term exposure to elevated CO_2 relative to winter wheat and winter rye. The cold acclimation-induced inhibition of CO_2 assimilation for spring wheat and spring rye at ambient CO_2 can not be compensated by a short-term exposure to elevated CO_2.

3.6 MATERIALS AND METHODS

3.6.1 Plant Materials and Growth Conditions

In all experiments, winter (cv Norstar) and spring (cv Katepwa) wheat (*Triticum aestivum L.*) and winter (cv Musketeer) and spring (cv SR4A) rye (*Secale cereale L.*) cultivars were used. Seeds were obtained from Agriculture and Agri-Food Canada, Indian Head Research Farm, Saskatchewan, Canada. Seeds were germinated and grown in the controlled environmental growth chambers (Model: GCW15 chamber, Environmental Growth Chambers, Chargin Falls, Ohio, USA) at ambient CO_2 of 380 ± 15 μmol C mol^{-1}, a PPFD of 250 ± 30 μmol photons m^{-2} s^{-1}, 50 - 60% relative humidity, a 16 h photoperiod and with day/night temperature regimes of 20/16°C for non-acclimated (NA) and 5/5°C for cold acclimated (CA) plants. The seedlings were grown in coarse vermiculite in 500mL-sized plastic pots at a density of three plants per pot and fertilized with Hoagland's solution.

To assess the effects of short-term elevated CO_2 on CO_2 gas exchange rates, the 25-day-old NA and 75-day-old CA plants grown at ambient CO_2 (380 μmol C mol^{-1}) were shifted to elevated CO_2 (700 μmol C mol^{-1}) for up to in specialized CO_2 chambers (Model: GCW15 chamber, Environmental Growth Chambers, Chargin Falls, Ohio, USA) with all other conditions remaining the same. CO_2 gas exchange rates were measured at different time points during the shift period at their respective growth temperatures.

To assess the temperature sensitivity of CO_2 gas exchange rates, the NA and CA plants were shifted for up to to varying day/night temperature regimes of either 5/5, 10/10, 20/20, 25/25, 30/30, 35/35 or 40/40°C at either ambient or elevated CO_2. CO_2 gas exchange rates were measured at different time points during the shift period at shifted temperatures and CO_2 levels.

Each CO_2 growth chamber was equipped with a computer-controlled CO_2 infra-red gas analyzer (Model: WMA-4 CO_2 Analyzer, PP Systems International, Inc. Amesbury, MA 01913 USA) which monitored CO_2 concentrations continuously such that elevated CO_2 concentrations were maintained at 700 ± 30 μmol C mol^{-1}. In addition, the temperature, relative humidity, irradiance level and photoperiod in each chamber were computer-controlled and monitored continuously.

3.6.2 Comparative Growth Kinetics

NA plants were harvested every week and CA plants every two weeks until the full stem elongation stage. The root and shoot fresh biomass were weighed. In order to determine dry weight, the root and shoot tissues were oven-dried at 80°C to constant weight. Exponential growth rates (g dry mass day^{-1}) were calculated from plots of the natural logarithm (ln) of shoot dry mass versus time in days. Specific leaf weight (SLW) was calculated as g leaf dry weight m^{-2} leaf blade area. Leaf blade area was measured by using a LI-COR portable area meter (LI–3000A, LI-COR Biosciences, Lincoln, Nebraska, USA).

3.6.3 CO2 Gas Exchange Measurements

CO_2 gas exchange rates were measured on fully expanded third leaves by using the LI-COR portable infrared CO_2 gas analyzer (LI-6400 XRT Portable Photosynthesis System, LI-COR Biosciences, Lincoln, NE, USA). Light response curves were measured by supplying 12 irradiance values over the range of 0 to 1500 μmol photons m^{-2} s^{-1} PPFD from high to low light intensity with 8 min of waiting time between each measurement. The apparent maximum quantum efficiency (Q) and the maximal photosynthetic capacity (A_{sat}) were determined as the maximum initial slope (25-200 μmol photons m^{-2} s^{-1}, Q) and the maximum light-saturated rates respectively from the light response curves. CO_2 response curves were measured by

supplying 11 different CO_2 values over the range of 50 to 1200 μmol C mol^{-1} at a saturating irradiance of 1000 to 1300 μmol photons m^{-2} s^{-1}. Rubisco-limited carboxylation efficiency (CE) was calculated as the maximum initial slope (50-300 μmol C mol^{-1}) of the curves. The maximum Rubisco carboxylation capacity (V_{cmax}) and the maximum rates of electron transport (J_{max}) were calculated using the FCB photosynthesis model (Farquhar *et al.,* 1980). Respiration rates (R_{dark}) were measured in the dark. Measurements were made inside the CO_2 enriched chambers to reduce diffusion errors through the leaf blade and chamber seals as cautioned by Jahnke and Krewitt (Jahnke 2001; Jahnke and Krewitt 2002). In addition, stomatal conductance and leaf transpiration rates were measured simultaneously with CO_2 gas exchange measurements. Leaf water use efficiency was calculated as the ratio of A_{sat} and stomatal conductance (A_{sat}/gs). All measurements for NA and CA plants were carried out on fully expanded third leaves at their respective growth temperatures.

3.6.4 Determination of total leaf protein and immunodetection

The fully expanded third leaves were harvested, immediately frozen in liquid nitrogen and stored at -80°C. The total leaf protein of the leaf tissues were extracted and quantified by using the RC-DC protein assay kit (Bio-Rad) according to the manufacturer's specifications.

The extracted proteins were electrophoretically separated using NuPAGE Novex 10% (w/v) Bis-Tris precast, polyacrylamide gels (Invitrogen) with MES SDS running buffer (Invitrogen) in an XCell4 SureLock Midi Cell (Invitrogen) according to manufacturer's specifications. Samples for SDS-PAGE were loaded on an equal protein basis (8 μg protein per lane). The addition of 1 μg of bovine serum albumin (Invitrogen) in the extraction buffer was used as an internal standard. Gels were electrophoresed and the separated polypeptides were electroblotted onto nitrocellulose membranes (0.2 μM pore size, Bio - Rad). The blots were blocked with

5% (w/v) fat free, dried milk powder overnight at 4°C and then probed with primary antibodies raised against the target proteins; rbcL, cFBPase, Lhcb1, psbA and psaA at a dilution of 1:2000-5000. The blots were probed with secondary antibody (anti-rabbit IgG Peroxidase antibody, Sigma-Aldrich) at a 1:10000-20000 dilutions (depending on the target proteins) and visualized with enhanced chemiluminesence immunodetection (ECL Detection Kit; GE Healthcare, UK) on X-ray film (Fujifilm, Fuji Corporation, Tokyo). Immunoblots were quantified by using a computer software program (Scion Image, Scion Corporation, Frederick, Maryland 21701, USA).

3.6.5 Analyses of gene expression

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out to achieve a wider view of the role of transcript to polypeptide changes that occurred during cold acclimation and short-term exposure to 40°C and/or elevated CO_2. The transcript levels for rbcL, cFBPase, Lhcb1, psbA and psaA were quantified by qRT-PCR. Total RNA was extracted from wheat leaves (200mg fresh weight) with Trizol reagent (Invitrogen). For qRT-PCR, 10 µg of total RNA was digested with DNASE I (Invitrogen) and half of the digested RNA was used for cDNA synthesis with random hexamers and Superscript Reverse Transcriptase (Invitrogen). The cDNAs obtained were diluted ten-fold in deionized water and 2 µl were used as template for real-time PCR. The polymerase chain reaction was performed with SYBR-Green PCR Mastermix (Invitrogen) and amplification was monitored on an ABI PRISM 7000 (Applied Biosystem). The 18S ribosomal gene was used as an internal standard for normalization of expression levels. The PCR amplification efficiency was 1.4 for all the genes tested. The primers used for the different genes are listed in Table S1 in supporting material.

3.6.6 Statistical Analysis

In all experiments, 20 replicate pots with three plants per pot for each cultivar in either NA or CA state were grown in a completely randomized design. Out of the 20 replicate pots, three pots for each cultivar at each growth condition were randomly selected for all photosynthetic measurements and biochemical analyses. Thus, all data are the averages of measurements made on nine different plants from three replicate pots. Results were subjected to analysis of variance (ANOVA). Means were compared at the 5% level of significance ($P \leq 0.05$) by using the statistical package SPSS version 17.

3.7 ACKNOWLEDGMENTS

This work was supported, in part, by the Natural Sciences and Engineering Research Council (NSERC) and industrial and government partners, through the Green Crop Research Network (GCN). NPAH, FS and BG also acknowledge research support through their individual NSERC Discovery Grants.

Table 3.1 : Effects of cold acclimation on exponential growth rates (EGR) and specific leaf weight (SLW) for winter and spring cereals. Samples were collected from fully expanded third leaves grown at ambient CO2 (380 µmol C mol-1) at either 20/16°C (NA) or 5/5°C (CA). Significant differences of the means between the acclimation state within each cultivar are indicated by the symbol * ($P \leq 0.05$). ± SD.

Cultivars	Acclimation state	EGR (g dry weight day^{-1})	CA/NA	SLW (g dry weight m^{-2})	CA/NA
Musketeer	NA	0.223 ± 0.041		34 ± 3	
	CA	0.072 ± 0.010*	0.323	79 ± 6*	2.32
Norstar	NA	0.215 ± 0.024		28 ± 4	
	CA	0.06 ± 0.008*	0.279	53 ± 6*	1.89
SR4A	NA	0.229 ± 0.029		35 ± 4	
	CA	0.075 ± 0.02*	0.328	42 ± 5	1.2
Katepwa	NA	0.212 ± 0.036		39 ± 2	
	CA	0.065 ± 0.014*	0.307	36 ± 5	0.92

Table 3.2: Effects of short-term elevated CO2 on the maximum Rubisco carboxylation capacity (Vcmax, µmol CO2 m-2 s-1), maximum rates of electron transport (Jmax, µmol e- m-2 s-1), the apparent maximum quantum efficiency (Q, CO2/photon) and the carboxylation efficiency (CE, CO2 m-2 s-1/ mol-1 CO2), for winter and spring cereals grown at ambient CO2 (380 µmol C mol-1) and at either 20/16°C (NA) or 5/5°C (CA). Measurements were carried out on fully expanded third leaves at 20°C. Significant differences among the means within each cultivar are indicated by the superscripted letters ($P \leq 0.05$). ± SD.

Cultivars	Acclimation	CO_2	V_{cmax}	J_{max}	Q	CE
Musketeer	NA	380	71 ± 4^{a}	157 ± 10^{a}	0.037 ± 0.002^{a}	0.072 ± 0.006^{a}
		700	75 ± 2^{a}	169 ± 8^{a}	0.053 ± 0.003^{b}	0.083 ± 0.009^{ab}
	CA	380	98 ± 7^{b}	220 ± 15^{b}	0.051 ± 0.002^{b}	0.098 ± 0.008^{b}
		700	93 ± 5^{b}	214 ± 19^{b}	0.056 ± 0.004^{b}	0.087 ± 0.006^{b}
Norstar	NA	380	67 ± 4^{a}	146 ± 10^{a}	0.053 ± 0.003^{a}	0.069 ± 0.004^{a}
		700	62 ± 3^{a}	142 ± 13^{a}	0.068 ± 0.004^{b}	0.073 ± 0.007^{ab}
	CA	380	79 ± 3^{b}	173 ± 9^{b}	0.057 ± 0.002^{a}	0.084 ± 0.004^{b}
		700	76 ± 6^{b}	164 ± 16^{ab}	0.059 ± 0.005^{ab}	0.077 ± 0.005^{ab}
SR4A	NA	380	84 ± 6^{b}	187 ± 14^{b}	0.049 ± 0.003^{b}	0.081 ± 0.005^{b}
		700	70 ± 5^{b}	168 ± 11^{b}	0.061 ± 0.004^{c}	0.078 ± 0.004^{b}

	CA	380	48 ± 3^{a}	121 ± 17^{a}	0.032 ± 0.004^{a}	0.062 ± 0.004^{a}
		700	53 ± 5^{a}	124 ± 8^{a}	0.036 ± 0.001^{a}	0.061 ± 0.008^{a}
Katepwa	NA	380	65 ± 8^{b}	161 ± 12^{a}	0.045 ± 0.002^{b}	0.087 ± 0.008^{b}
		700	60 ± 4^{b}	151 ± 10^{a}	0.058 ± 0.004^{c}	0.075 ± 0.010^{b}
	CA	380	37 ± 2^{a}	99 ± 7^{a}	0.034 ± 0.002^{a}	0.049 ± 0.004^{a}
		700	31 ± 3^{a}	89 ± 9^{a}	0.034 ± 0.003^{a}	0.053 ± 0.006^{a}

Table 3.3: Effects of short-term shift to elevated CO2 on stomatal conductance, transpiration rates and water use efficiency (WUE) for winter and spring cereals grown at ambient CO2 (380 μmol C mol-1) and at either 20/16°C (NA) or 5/5°C (CA). Measurements were carried out on fully expanded third leaves at their respective growth CO2 and at 20°C. Significant differences among the means within each cultivar are indicated by the superscripted letters ($P \leq 0.05$). ± SD.

Cultivars	Acclimation	CO_2 (μmol C mol^{-1})	Stomatal Conductance gs (mol m^{-2} s^{-1})	Transpiration (mmol H_2O m^{-2} s^{-1})	WUE (A/gs)
Musketeer	NA	380	0.45 ± 0.04^{b}	2.65 ± 0.30^{b}	38 ± 6^{a}
		700	0.26 ± 0.03^{a}	2.02 ± 0.26^{a}	108 ± 14^{bc}
	CA	380	0.28 ± 0.02^{a}	1.79 ± 0.12^{a}	84 ± 9^{b}
		700	0.31 ± 0.03^{a}	1.82 ± 0.35^{a}	136 ± 10^{c}
Norstar	NA	380	0.51 ± 0.04^{b}	3.42 ± 0.24^{b}	30 ± 4^{a}
		700	0.30 ± 0.03^{a}	2.32 ± 0.36^{a}	80 ± 15^{bc}
	CA	380	0.24 ± 0.02^{a}	1.76 ± 0.41^{a}	72 ± 7^{b}
		700	0.28 ± 0.02^{a}	1.94 ± 0.21^{a}	107 ± 12^{c}

SR4A	NA	380	0.62 ± 0.05^{b}	3.09 ± 0.22^{b}	32 ± 8^{a}
		700	0.37 ± 0.03^{a}	2.25 ± 0.37^{a}	85 ± 13^{c}
	CA	380	0.40 ± 0.03^{a}	2.39 ± 0.29^{a}	37 ± 4^{a}
		700	0.36 ± 0.02^{a}	2.15 ± 0.12^{a}	56 ± 8^{b}
Katepwa	NA	380	0.39 ± 0.03^{c}	3.24 ± 0.49^{c}	46 ± 6^{a}
		700	0.23 ± 0.02^{ab}	2.01 ± 0.32^{a}	128 ± 18^{c}
	CA	380	0.27 ± 0.02^{b}	2.56 ± 0.17^{b}	43 ± 3^{a}
		700	0.18 ± 0.04^{a}	2.17 ± 0.27^{a}	74 ± 10^{b}

Table 3.4 : Effects of short-term shift to elevated CO2 and /or high temperature on leaf protein content for winter and spring cereals grown at ambient CO2 (380 µmol C mol-1) and at either 20/16°C (NA) or 5/5°C (CA). Samples were collected from 25-day-old NA and 75-day-old CA plants; at ambient CO2 and respective growth temperatures, and after 80 h at (i) elevated CO2 (700 µmol C mol-1) at constant temperature (ii) ambient CO2 at 40°C and (iii) elevated CO2 at 40°C. Significant differences among the means within each cultivar are indicated by the superscripted letters ($P \leq 0.05$). ± SD.

		Total Leaf protein Content (g m^{-2} leaf area)			
			80 h shift to		
Cultivars	Acclimation state	Ambient CO_2	Elevated CO_2	Ambient CO_2 + 40°C	Elevated CO_2 + 40°C
Musketeer	NA	2.70 ± 0.46^{b}	2.40 ± 0.31^{b}	1.12 ± 0.14^{a}	1.08 ± 0.19^{a}
	CA	12.65 ± 2.20^{d}	11.97 ± 1.70^{d}	7.41 ± 1.16^{c}	7.95 ± 1.30^{c}
Norstar	NA	2.32 ± 0.31^{b}	2.14 ± 0.39^{b}	1.10 ± 0.16^{a}	0.98 ± 0.10^{a}
	CA	9.84 ± 1.60^{d}	9.30 ± 1.20^{d}	5.89 ± 0.66^{c}	5.15 ± 0.89^{c}
SR 4A	NA	3.33 ± 0.54^{bc}	3.05 ± 0.29^{b}	1.91 ± 0.30^{a}	1.78 ± 0.22^{a}
	CA	4.01 ± 0.37^{c}	3.77 ± 0.26^{c}	2.30 ± 0.34^{a}	2.17 ± 0.38^{a}
Katepwa	NA	2.89 ± 0.45^{bc}	2.53 ± 0.22^{b}	1.49 ± 0.26^{a}	1.80 ± 0.31^{a}
	CA	3.27 ± 0.27^{c}	2.74 ± 0.40^{bc}	1.60 ± 0.33^{a}	1.78 ± 0.21^{a}

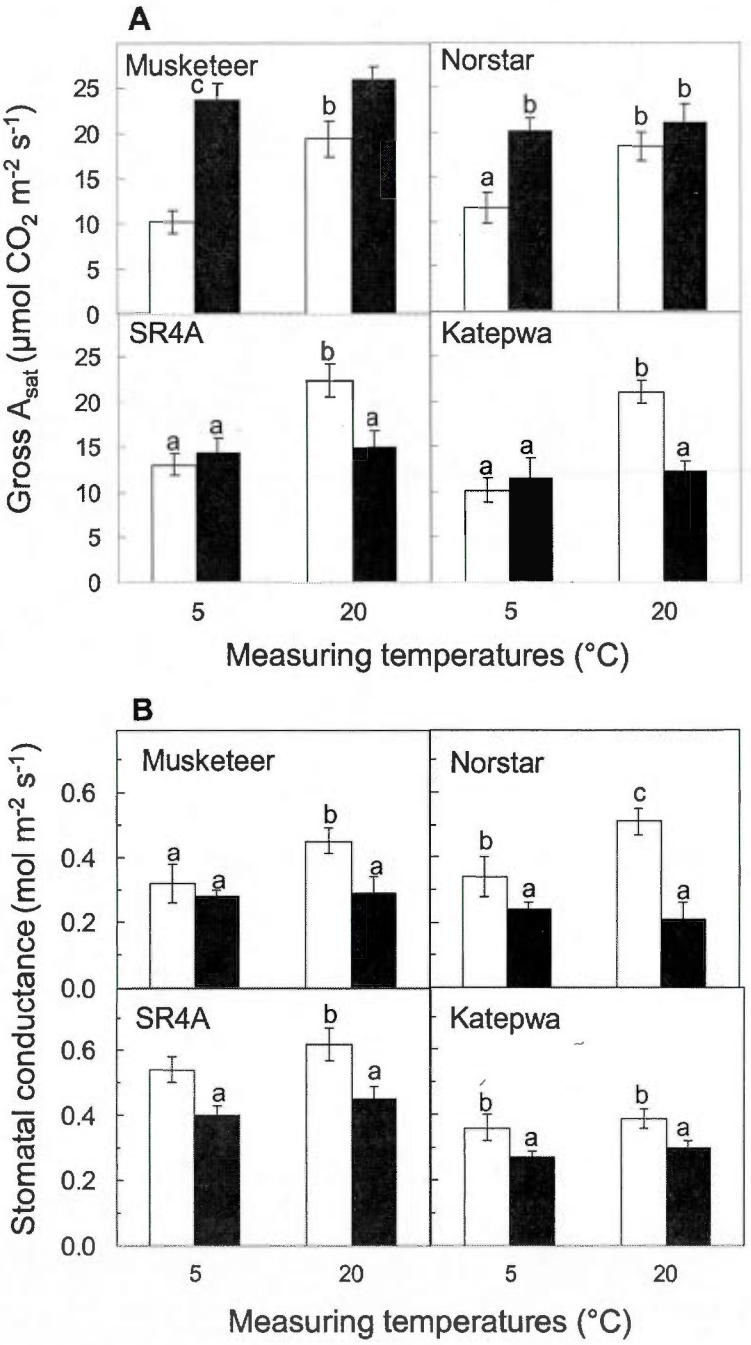

Figure 3.1 : Effects of measuring temperatures on (A) gross Asat and (B) stomatal conductance of NA (□) and CA (■) winter and spring cereals. Bars represent SD.

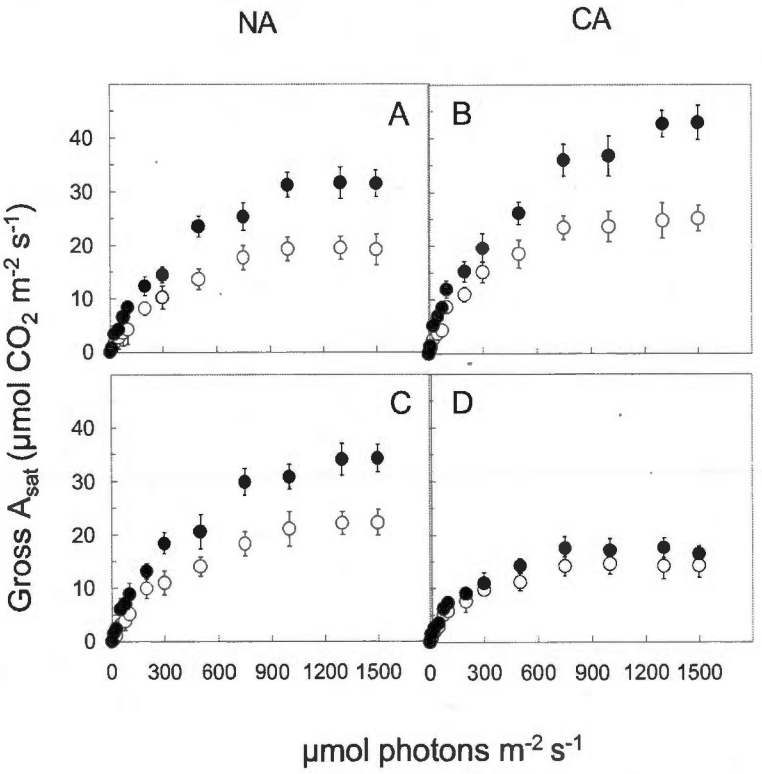

Figure 3.2: Light response curves of gross CO2 assimilation at either ambient (O) or after 80 h of exposure to elevated (●) CO2 for NA (A, C) and CA (B, D) Musketeer (A, B) and SR4A (C, D) rye. Bars represent

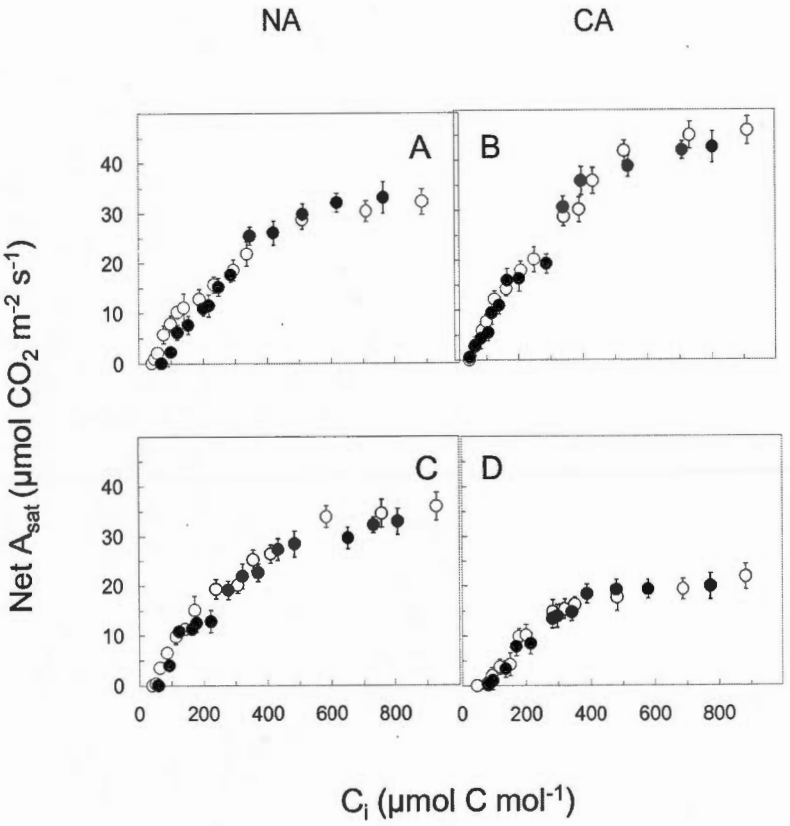

Figure 3.3: CO2 response curves of light-saturated net CO2 assimilation at either ambient (O) or after 80 h of exposure to elevated (●) CO2 for NA (A, C) and CA (B, D) Musketeer (A, B) and SR4A (C, D) rye. Bars represent SD.

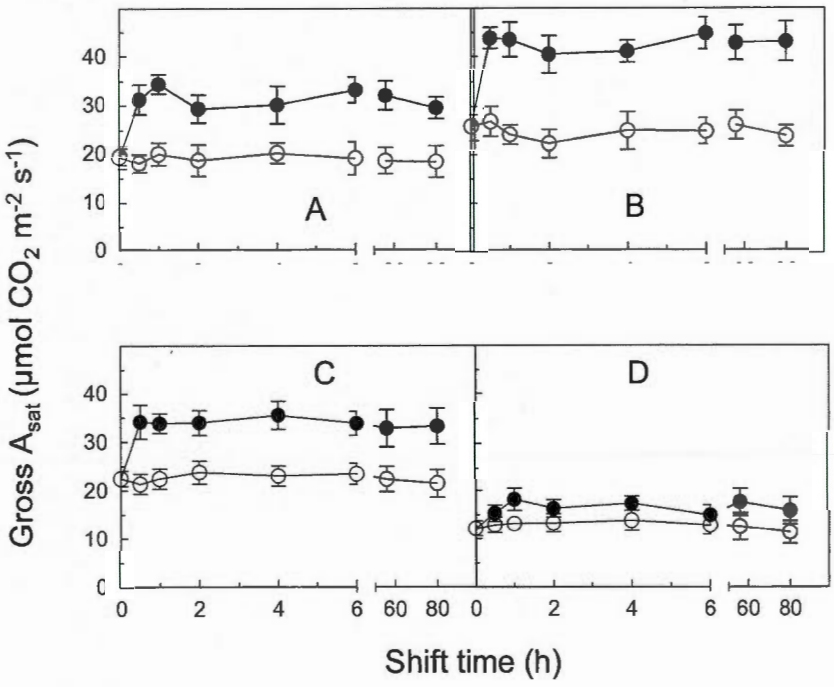

Figure 3.4 : Light-saturated rates of gross CO2 assimilation at either ambient (O) or over 80 h of exposure to elevated (●) CO2 for NA (A, C) and CA (B, D) Musketeer (A, B) and SR4A (C, D) rye. Bars represent SD.

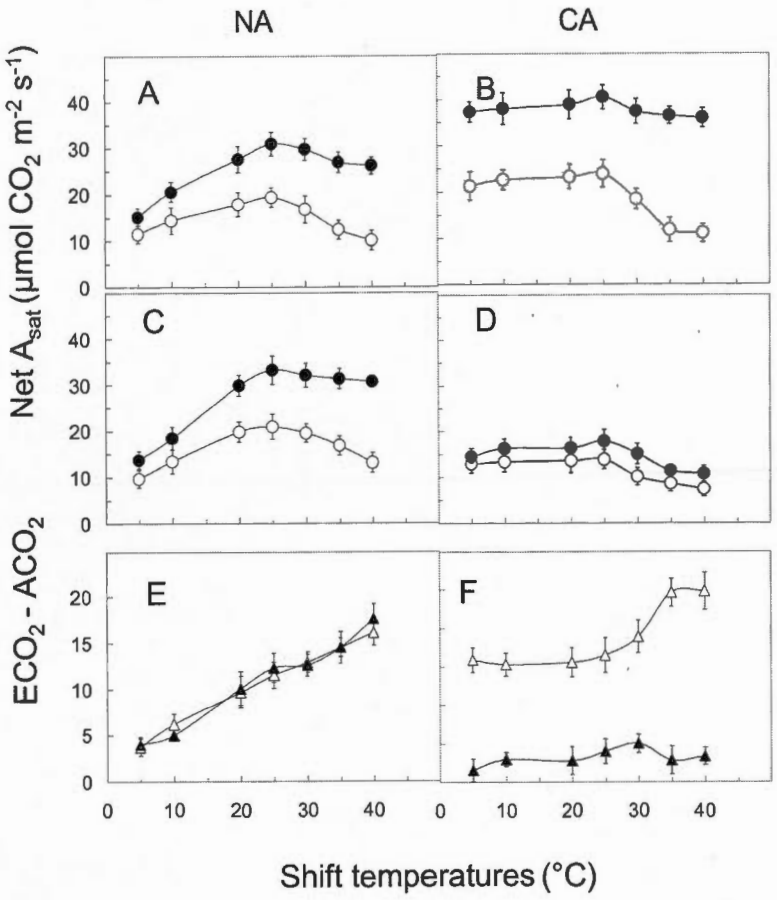

Figure 3.5: Temperature sensitivity of net Asat at either ambient (O) or after 1 h shift to elevated (●) CO2 for NA (A, C,) and CA (B, D) Musketeer (A, B) and SR4A (C, D) rye. Difference in net Asat at elevated versus ambient CO2 (ECO2 - ACO2) for NA (E) and CA (f) Musketeer (Δ) and SR4A (▲). Bars represent SD.

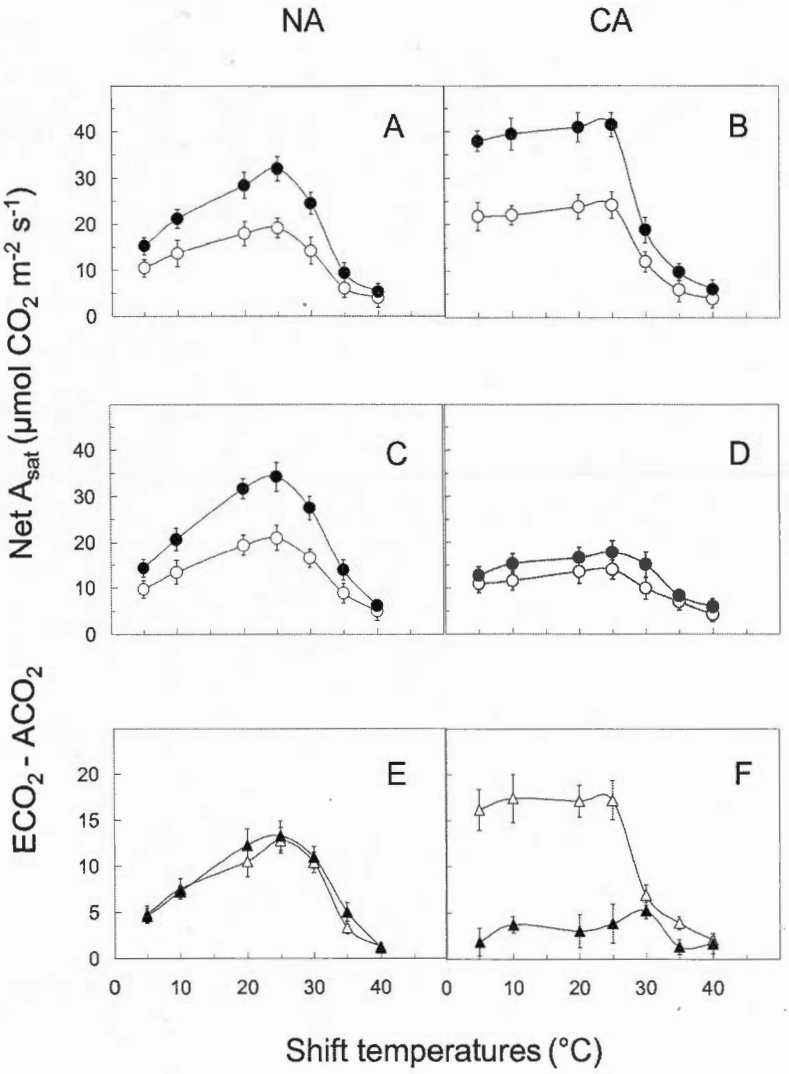

Figure 3.6: Temperature sensitivity of net Asat at either ambient (O) or after 80 h shift to elevated (●) CO2 for NA (A, C) and CA (B, D) Musketeer (A, B) and SR4A (C, D) rye. Difference in net Asat at elevated versus ambient CO2 (ECO2 - ACO2) for NA (E) and CA (F) Musketeer (Δ) and SR4A (▲). Bars represent SD.

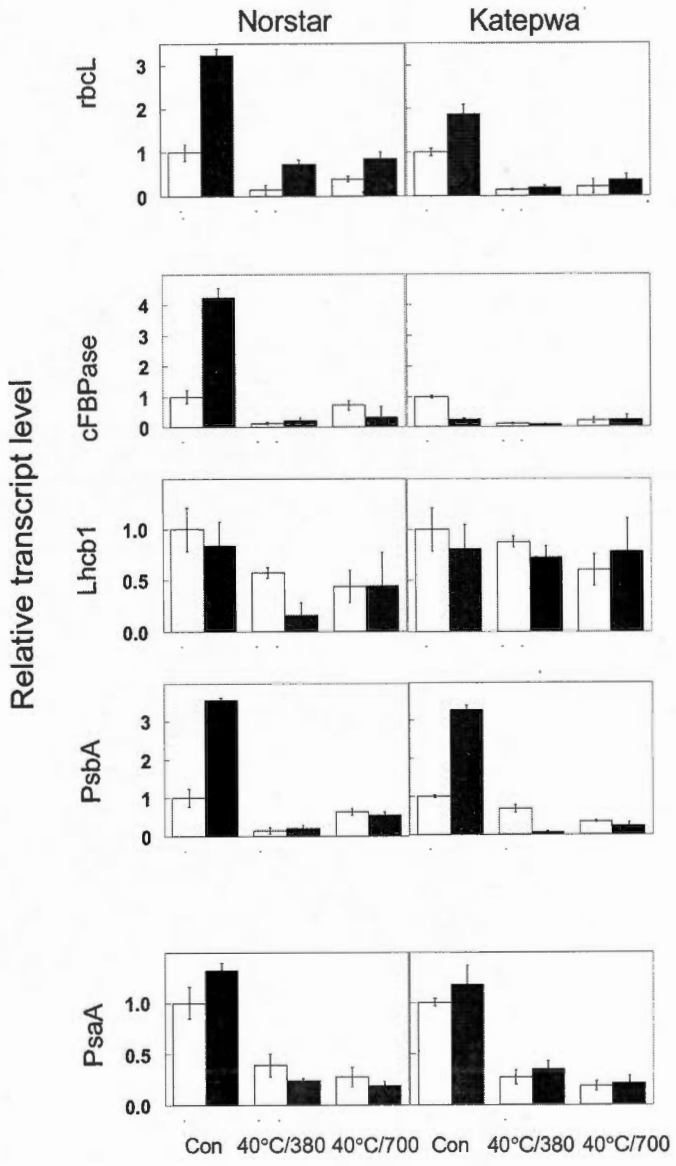

Figure 3.7: Effects of high temperature (40°C) and elevated CO2 (700 µmol C mol-1) on transcript levels of photosynthetic genes isolated from NA (□) and CA (■) Norstar and Katepwa wheat. Bars represent SD. Con = controls (growth temperature and CO2).

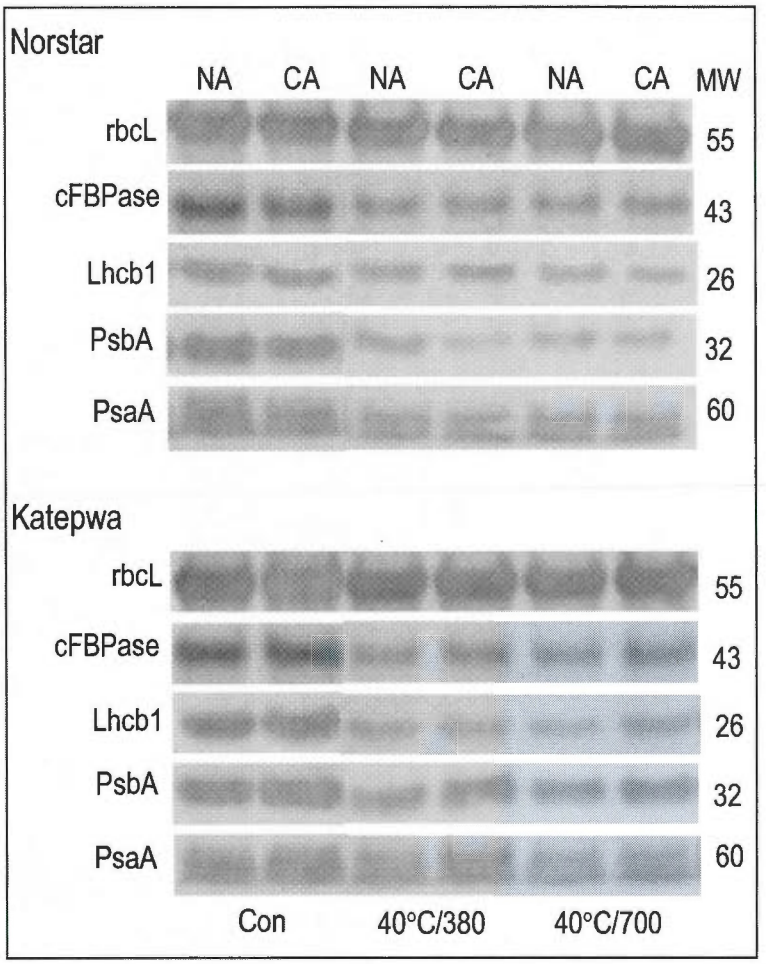

Figure 3.8: Effects of high temperature (40°C) and elevated CO2 (700 μmol C mol-1) on photosynthetic proteins isolated from NA and CA Norstar and Katepwa wheat. Numbers on the right indicate molecular masses (kDa) of markers. Con = controls (growth temperature and CO2).

3.8 SUPPORTING INFORMATION

Table 3.S1: List of primers used to analyze the transcript levels of photosynthetic genes isolated from Norstar and Katepwa wheat.

Genes	Sequences
rbcL	5'-CTA CGC GGT GGA CTT GAT TT-3'
	3'-ATT TCA CCA GTT TCG GCT TG-5'
cFBPase	5'-AAC AAG AAC GAG GGA GGG ATA C-3'
	3'-TCC GCA TCA CAA GAA AAG G-5'
Lhcb1	5'-CGT CCT TCG GAC AAA TAT GC-3'
	3'-TAA TGA CAT GGG CCA GCA AG-5'
PsbA	5'-GTG GCT GCT CAC GGT TAT TT-3'
	3'-CCA AGC AGC CAA GAA GAA GT-5'
PsaA	5'-GGA AAA TGC AGT CGG ATG TT-3'
	3'-AGA AAT CTC GAA GCC AAC CA-5'

Table 3.S2: Effects of pot size on dry matter accumulation of Musketeer winter rye grown at ambient CO2. ± SD.

	Total DW mg plant $^{-1}$	
Pot size (L)	NA	CA
0.5	331 ± 45	307 ± 29
2	349 ± 22	324 ± 33
4	366 ± 31	348 ± 39
6	352 ± 37	331 ± 21

Table 3.S3: Effects of short-term shift to elevated CO2 and /or high temperature on dark respiratory rates for winter and spring cereals grown at ambient CO2 (380 µmol C mol-1) and at either 20/16°C (NA) or 5/5°C (CA). Respiratory rates for NA and CA plants were measured at ambient CO2 and respective growth temperatures, and after 80 h at (i) elevated CO2 (700 µmol C mol-1) at constant temperature (ii) ambient CO2 at 40°C and (iii) elevated CO2 at 40°C. Significant differences among the means within each cultivar are indicated by the superscripted letters ($P \leq 0.05$). ± SD.

		Respiration (µmol CO_2 evolved m^{-2} s^{-1})			
			80 h shift to		
Cultivars	Acclimation state	Ambient CO_2	Elevated CO_2	Ambient CO_2 + 40°C	Elevated CO_2 + 40°C
Musketeer	NA	3.24 ± 0.14^{c}	2.83 ± 0.13^{b}	1.51 ± 0.24^{a}	1.37 ± 0.08^{a}
	CA	3.88 ± 0.23^{d}	3.13 ± 0.29^{bc}	1.22 ± 0.11^{a}	1.31 ± 0.16^{a}
Norstar	NA	2.28 ± 0.11^{c}	2.14 ± 0.39^{c}	1.30 ± 0.28^{ab}	0.98 ± 0.13^{a}
	CA	2.85 ± 0.18^{d}	2.38 ± 0.20^{c}	1.39 ± 0.21^{b}	1.35 ± 0.10^{b}
SR 4A	NA	3.05 ± 0.12^{d}	2.54 ± 0.18^{c}	1.77 ± 0.12^{b}	1.41 ± 0.20^{ab}
	CA	3.43 ± 0.13^{e}	2.89 ± 0.21^{cd}	1.35 ± 0.16^{a}	1.51 ± 0.35^{ab}
Katepwa	NA	2.07 ± 0.24^{bc}	1.63 ± 0.28^{b}	1.28 ± 0.19^{a}	1.26 ± 0.11^{a}
	CA	2.35 ± 0.29^{c}	2.31 ± 0.17^{c}	1.12 ± 0.14^{a}	1.23 ± 0.22^{a}

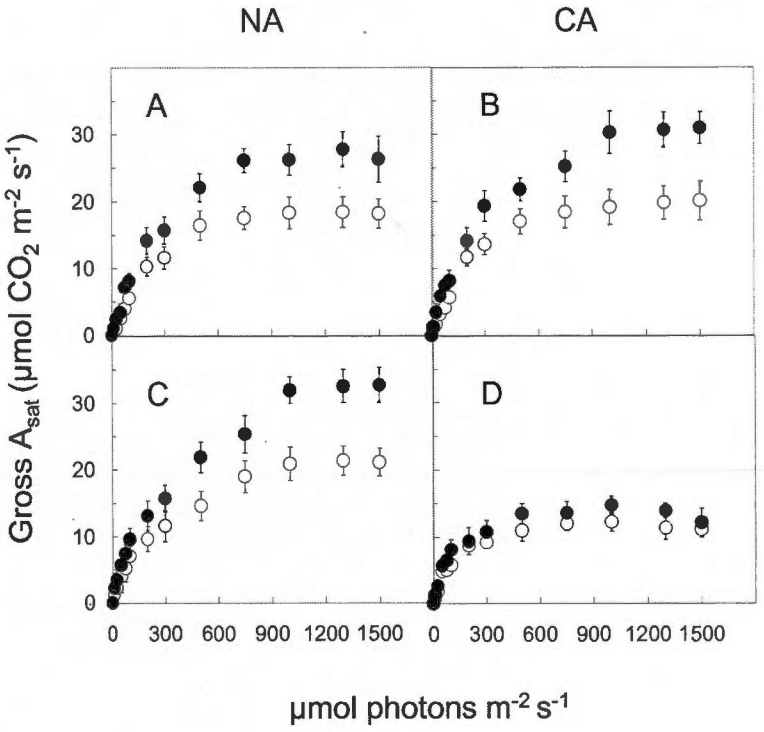

Figure 3.S1: Light response curves of gross CO2 assimilation at either ambient (O) or after 80 h of exposure to elevated (●) CO2 for NA (A, C) and CA (B, D) Norstar (A, B) and Katepwa (C, D) wheat. Bars represent SD.

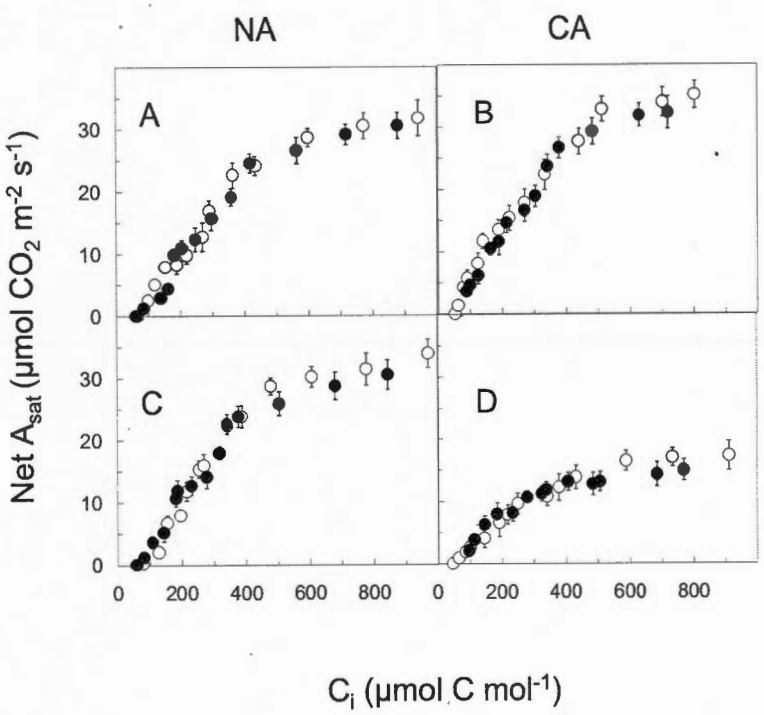

Figure 3.S2: CO2 response curves of light-saturated net CO2 assimilation at either ambient (O) or after 80 h of exposure to elevated (●) CO2 for NA (A, B) and CA (C, D) Norstar (A, B) and Katepwa (C, D) wheat. Bars represent SD.

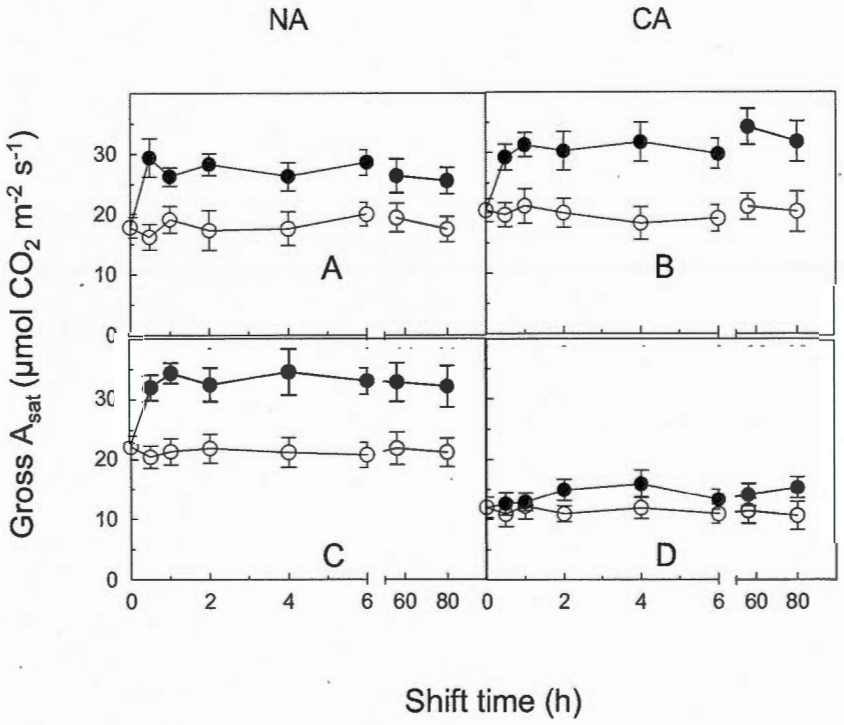

Figure 3.S3: Light-saturated rates of gross CO2 assimilation at either ambient (O) or over 80 h of exposure to elevated (●) CO2 for NA (A, C) and CA (B, D) Norstar (A, B) and Katepwa (C, D) wheat. Bars represent SD.

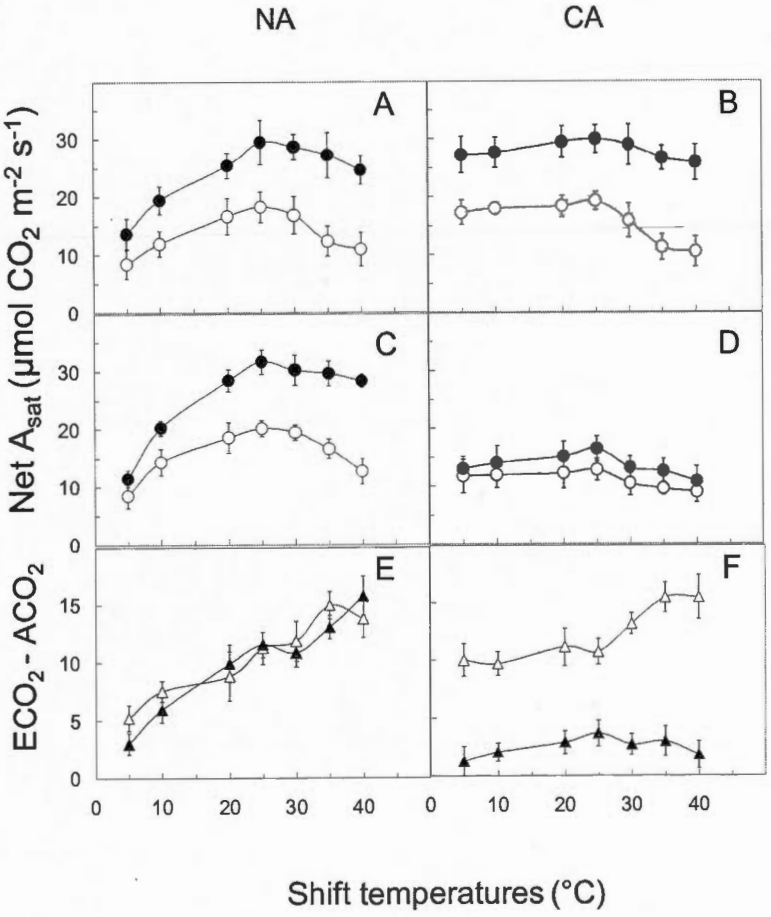

Figure 3.S4: Temperature sensitivity of net Asat at either ambient (O) or after 1 h shift to elevated (●) CO2 for NA (a, c,) and CA (b, d) Norstar (a, b) and Katepwa (c, d) wheat. Difference in net Asat at elevated versus ambient CO2 (ECO2 - ACO2) for NA (e) and CA (f) Norstar (Δ) and Katepwa (▲). Bars represent SD.

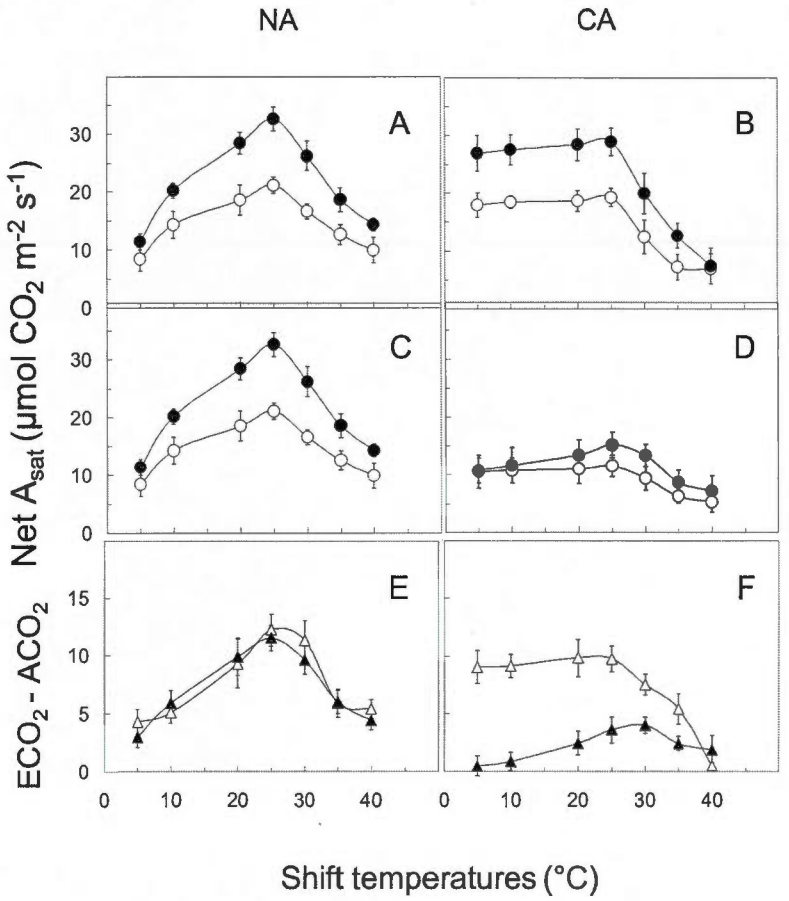

Figure 3.S5: Temperature sensitivity of net Asat at either ambient (O) or after 80 h shift to elevated (●) CO2 for NA (a, c,) and CA (b, d) Norstar (a, b) and Katepwa (c, d) wheat. Difference in net Asat at elevated versus ambient CO2 (ECO2 - ACO2) for NA (e) and CA (f) Norstar (Δ) and Katepwa (▲). Bars represent SD.

3.9 REFERENCES

Adams III, W.W., Demmig-Adams, B., Rosenstiel, T.N., Brightwell, A.K., and Ebbert, V. 2002. Photosynthesis and photoprotection in overwintering plants. Plant Biol. **4**(5): 545-557.

Ainsworth, E.A., and Rogers, A. 2007. The response of photosynthesis and stomatal conductance to rising CO_2: mechanisms and environmental interactions. Plant Cell Environ. **30**(3): 258-270.

Boese S.R., and Hüner N.P.A. 1992. Developmental history affects the susceptibility of spinach leaves to in vivo low temperature photoinhibition. Plant Physiol. **99**(3): 1141-1145.

Cheng, S.H., Moore, B., and Seemann, J.R. 1998. Effects of short and long-term elevated CO_2 on the expression of Ribulose-1,5-bisphosphate carboxylase/oxygenase genes and carbohydrate accumulation in leaves of *Arabidopsis thaliana* (L.) Heynh. Plant Physiol. **116**(2): 715-723.

Dahal, K., Kane, K., Gadapati, W., Webb, E., Savitch, L.V., Singh, J., Sharma, P., Sarhan, F., Longstaffe, F.J., Grodzinski, B., Hüner, N.P.A. 2011. The Effects of Phenotypic Plasticity on Photosynthetic Performance in Winter Rye, Winter Wheat and *Brassica napus*. Physiol Plant. **144** (2) 169-188.

Ensminger, I., Busch, F., and Hüner, N.P.A. 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol. Plant. **126**(1): 28-44.

Farquhar, G.D., Caemmerer, S., and Berry, J.A. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta **149**:78-90.

Gray, G.R., Savitch, L.V., Ivanov, A., and Hüner N.P.A. 1996. Photosystem II excitation pressure and development of resistance to photoinhibition II. Adjustment of photosynthetic capacity in winter wheat and winter rye. Plant Physiol. **110**(1): 61-71.

Hüner, N.P.A. 1985. Morphological, anatomical and molecular consequences of growth and development at low temperature in *Secale ereale* L. cv Puma. Am. J. Bot. **72**: 1290-1306.

Hüner, N.P.A., Palta, J.P., Li, P.H., and Carter, J.V. 1981. Anatomical changes in leaves of Puma rye in response to growth at cold hardening temperatures. Bot. Gaz. **142**(1): 55-62.

Hüner, N.P.A., Öquist, G., Hurry, V.M., Krol, M., Falk, S., and Griffith, M. 1993. Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. Photosyn Res **37**(1): 19-39.

Hüner, N.P.A., Öquist, G., and Sarhan, F. 1998. Energy balance and acclimation to light and cold. Trends Plant Sci. **3**(6): 224-230.

Hurry, V.M., and Hüner, N.P.A. 1991. Low growth temperature effects a differential inhibition of photosynthesis in spring and winter wheat. Plant Physiol. **96**(2): 491-497.

Hurry, V.M., Malmberg, G., Gardeström, P., and Öquist, G. 1994. Effects of a short-term shift to low temperature and of long-term cold hardening on photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase and sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). Plant Physiol. **106**(3): 983-990.

Hurry, V.M., Strand, A., Tabiaeson, M., Gardeström, P., and Öquist, G. 1995. Cold hardening of spring and winter wheat and rape results in differential effects on growth, carbon metabolism, and carbohydrate content. Plant Physiol. **109**(2): 697-706.

Jahnke, S. 2001. Atmospheric CO_2 concentration does not directly affect leaf respiration in bean or poplar. Plant Cell Environ. **24**(11): 1139–1151.

Jahnke, S., and Krewitt, M. 2002. Atmospheric CO_2 concentration may directly affect leaf respiration measurement in tobacco, but not respiration itself. Plant Cell and Environ. **25**(5): 641–651.

Krause, G.H. 1988. Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. Physiol. Plant. **74**(3): 566-574.

Kumar, A., Li, C., and Portis, A.R. 2009. Arabidopsis thaliana expressing a thermostable chimeric Rubisco activase exhibits enhanced growth and higher rates of photosynthesis at moderately high temperatures. Photosyn. Res. **100**(3): 143-153.

Leonardos, E.D., Savitch, L.V., Hüner, N.P.A., Öquist, G., and Grodzinski, B. 2003. Daily photosynthetic and C-export patterns in winter wheat leaves during cold stress and acclimation. Physiol. Plant. **117**(4): 521-531.

Long, S.P., Ainsworth, E.A., Rogers, A., and Ort, D.R. 2004. Rising atmospheric carbon dioxide: plants FACE the future. Ann. Rev. Plant Biol. **55**: 591-628.

Öquist, G., and Hüner, N.P.A. 2003. Photosynthesis of overwinteering evergreen plants. Ann. Rev. Plant Biol. **54**: 329-355.

Öquist, G., Hurry, V.M., and Hüner, N.P.A. 1993. Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. Plant Physiol. **101**(1): 245-250.

Pocock, T.H., Hurry, V.M., Savitch, L.V., and Hüner, N.P.A. 2001. Susceptibility to low-temperature photoinhibition and the acquisition of freezing tolerance in winter and spring wheat: The role of growth temperature and irradiance. Physiol. Plant. **113**(4): 499-506.

Rapacz, M., Wolanin, B., Hura, K., and Tyrka, M. 2008. The effects of cold acclimation on photosynthetic apparatus and the expression of COR14b in four genotypes of barley (Hordeum vulgare) contrasting in their tolerance to freezing and high-light treatment in cold conditions. Annals Bot. **101**(5): 689–699.

Salvucci, M.E., and Crafts-Brandner, S.J. 2004. Inhibition of photosynthesis by heat stress: the activation state of Rubisco as a limiting factor in photosynthesis. Physiol. Plant. **120**(2): 179–186.

Sarhan, F., Ouellet, F., and Vazquez-Tello, A. 1997. The wheat *Wcs120* gene family: a useful model to understand the molecular genetics of freezing tolerance in cereals. Physiol. Plant. **101**(2): 439–445.

Sassenrath, G.F., and Ort, D.R. 1990. The relationship between inhibition of photosynthesis at low temperature and the inhibition of photosynthesis after rewarming in chill-sensitive tomato. Plant Physiol. Biochem. **28**: 457-465.

Savitch, L.V., Barker-Astrom, J., Ivanov, A. G., Hurry, V., Oquist, G., Hüner, N.P.A. 2001. Cold acclimation of Arabidopsis thaliana results in incomplete recovery of photosynthetic capacity which is associated with an increased reduction of the chloroplast stroma. Planta **214**(2): 295-301.

Savitch, L.V., Leonardos, E.D., Krol, M., Jansson, S., Grodzinski, B., Hüner, N.P.A., and Öquist, G. 2002. Two different strategies for light utilization in photosynthesis in relation to growth and cold acclimation. Plant Cell Environ. **25**(6): 761-771.

Somersalo, S., and Krause, G.H. 1989. Photoinhibition at chilling temperatures: Fluorescence characteristics of unhardened and cold-hardened spinach leaves. Planta **177**: 409-416.

Stitt, M., and Hurry, V.M. 2002. A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Curr. Opin. Plant Biol. **5**(3): 199-206.

Strand, A., Hurry, V.M., Henkes, S., Hüner, N.P.A., Gustafsson, P., Gardeström, P., and Stitt, M. 1999. Acclimation of Arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol. **119**(4): 1387-1398.

Tcherkez, G.G.B., Farquhar, G.D., and Andrews, T.J. 2006. Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. Proc. Nat. Acad. Sci. **103**(19): 7246–7251.

CHAPITRE IV

ARTICLE III: LONG TERM GROWTH UNDER ELEVATED CO2 DIFFERENTIALLY SUPPRESSES BIOTIC STRESS GENES IN NON ACCLIMATED VERSUS COLD ACCLIMATED WINTER WHEAT

Khalil Kane, Keshav Dahal, Mohamed Badawi, Mario Houde, Norman P.A. Hüner and Fathey Sarhan

Contribution

J'ai participé dans plusieurs étapes de l'expérimentation et de rédaction de l'article. J'ai effectué les analyses bioinformatiques des puces à ADN avec Mario Houde. J'ai également effectué la classification des gènes régulés par le CO_2 et le froid en utilisant les outils bioinformatiques tels que décrits dans l'article. Finalement j'ai validé l'expression des gènes régulés par les analyses de PCR en temps réel. Cet article a été soumis dans la revue plant physiology.

4.1 RÉSUMÉ

La hausse de la concentration de CO_2 prévue d'ici la fin du siècle va avoir des effets drastiques sur la majorité des cultures. L'analyse du transcriptome chez le blé (*Triticum aestivum*. L,) d'hiver Norstar cultivé à long terme en conditions de CO_2 ambiant (380 µmol C mol^{-1}) et élevé (700 µmol C mol^{-1}) a été initiée afin de déterminer les facteurs physiologiques et génétiques impliqués dans la réponse des plantes non-acclimatés (NA, 20°C) et acclimaté au froid (CA, 5 °C) aux concentrations élevées de CO_2. Les plantes acclimatées au froid en conditions de CO_2 ambiant présentent, un phénotype court et robuste, une réduction de 33% de leur croissance, une hausse double du poids spécifique de leur feuille de même que de leur quantité de protéine et une hausse de 30% de leur quantité de chlorophylle par unité de surface foliaire comparativement aux plantes non acclimaté. Les concentrations élevées de CO_2 ont peu d'effets sur ces paramètres morphologiques. Néanmoins les concentrations élevées de CO_2 ont entraîné une hausse de 30% de la biomasse des parties aériennes du blé non acclimaté et acclimaté au froid. Le blé Norstar acclimaté au froid maintient des taux de photosynthèses en conditions de lumière et de CO_2 saturantes comparables au blé non-acclimaté, mais requiert moins de quantum pour la fermeture du photosystème et pour la dissipation de l'énergie sous forme de chaleur. Les plantes non acclimaté et acclimaté au froid ne sont pas sensibles à l'inhibition photosynthétique induite par les concentrations élevées de CO_2. L'effet le plus marqué des concentrations élevées de CO_2 chez le blé non acclimaté est la diminution de l'expression des gènes impliqués dans la défense des plantes face aux pathogènes. Par contre, ces effets sont moins accentués chez les plantes acclimatées au froid grâce à l'induction des gènes impliqués dans la résistance aux pathogènes, dans la tolérance au gel, dans la protection et à la stabilisation du chloroplaste. Ces résultats démontrent que l'acclimatation au froid et les concentrations élevées de CO_2 ont des effets opposés sur la régulation du système de défense des plantes. Ces résultats démontrent que es concentrations élevées de CO2 auront moins d'impact sur la performance et la productivité des plantes qui vivent dans les pays nordiques contrairement à celles qui vivent dans les environnements plus chauds. Avec les conditions de CO_2 élevé prévu, la sélection de plantes ayant des caractères de résistance aux pathogènes est fortement suggérée.

4.2 ABSTRACT

Rising atmospheric CO_2 concentration during the century will have a drastic effect on major crops. A transcriptomic analysis of winter wheat *Triticum aestivum* L. cv Norstar during long-term growth under ambient (AC, 380 μmol C mol^{-1}) and elevated CO_2 (EC, 700 μmol C mol^{-1}) was initiated to determine the physiological and genetic factors involved in plant responses to EC during non acclimated (NA, 20°C) and cold acclimated (CA, 5 °C) conditions. Although cold acclimation at AC induced a dwarf phenotype, reduced exponential growth rates by about 33%, but increased leaf specific weight as well as leaf protein per leaf area by 2-fold and chlorophyll per leaf area by 30% relative to NA Norstar grown to a comparable developmental state, EC had minimal effects on these morphological characteristics. However, EC increased total shoot biomass by about 30% under both NA and CA conditions. CA Norstar maintained comparable light saturated and CO_2 saturated rates of photosynthesis but lower quantum requirements for photosystem II closure and non photochemical quenching relative to NA plants even after growth at EC. NA and CA plants were not sensitive to feedback inhibition of photosynthesis at EC. The most striking effect was the down-regulation of genes involved in the plant defense responses in NA Norstar. In contrast, down regulation of plant defense genes was minimal in CA plants due to the induction of genes involved in plant pathogenesis resistance, freezing tolerance, protection and stabilization of chloroplasts. Thus EC may have less impact on plant performance and productivity in northern climates compared to warmer environments. We suggest that selection for cultivars with constitutively higher expression of biotic stress defense genes in cereal crops may be necessary under EC.

4.3 INTRODUCTION

Carbon dioxide is one the most important green house gases responsible for global warming. Predictions by the Intergovernmental Panel on Climate Change estimate that the actual atmospheric concentration of CO_2 will double by the end of the century (Houghton *et al.,* 2001; Solomon *et al.,* 2007). It has been established that short–term exposure of C_3 plants from ambient to elevated CO_2 (EC) results in an increase in the rates of net CO_2 assimilation (Drake *et al.,* 1997; Farquhar 1997; Chen *et al.,* 2005; Long *et al.,* 2004; Ainsworth and Rogers 2007; Dahal *et al.,* 2012c). This stimulation of photosynthesis in C_3 plants is due to two major reasons. First, the K_m (CO_2) for Rubisco is close to the current atmospheric CO_2 (Long *et al.,* 2004, Tcherkez *et al.,* 2006) which indicates that Rubisco is CO_2 substrate-limited at ambient CO_2. Thus, an immediate increase in carboxylation velocity is expected by increased CO_2 substrate availability. Second, EC competitively inhibits the light-dependent evolution of CO_2 by photorespiration because CO_2 is a competitive inhibitor of the oxygenation of RuBP by Rubisco (Long *et al.,* 2004). The observation of enhanced CO_2 assimilation in response to short-term CO_2 exposure is consistent for experiments conducted in controlled environment chambers as well as in free-air CO_2 enrichment (FACE) experiments although the extent of the observed stimulation on CO_2 assimilation rates can vary between the two experimental designs (Ainsworth and Rogers 2007; Li *et al.,* 2008).

Acclimation to long-term exposures to EC in C3 plants is generally associated with a suppression of carbon metabolism favouring carbon accumulation in the form of soluble carbohydrates and starch coupled with the down-regulation of genes involved in nitrogen metabolism (Delucia *et al.,* 1985; Drake *et al.,* 1997; Lee *et al.,* 2001; Bloom *et al.,* 2002; Ainsworth and Long, 2005). Long-term growth and development of C_3 plants at high CO_2 concentrations may lead to an end product inhibition of photosynthetic capacity due to accumulation of carbohydrates in the

cytosol (Stitt and Quick 1989; Foyer *et al.,* 1990). This feedback inhibition of photosynthesis in response to growth at EC may result from the chloroplastic P_i-limitation in the short-term and down-regulation of the expression and activities of key regulatory photosynthetic enzymes in the long-term (Harley and Sharkey 1991; Drake *et al.,* 1997; Moore *et al.,* 1998).

Transcriptomic studies associated with long term acclimation to EC have been performed using microarrays in species such as poplar, soybean, Arabidopsis, rice and strawberry (Taylor *et al.,* 2005; Ainsworth *et al.,* 2006; Ainsworth *et al.,* 2008; Fukayama *et al.,* 2009; Ponce-Valadez *et al.,* 2009). In *Arabidopsis*, exposure to long-term EC down-regulated photosynthesis as indicated by the accumulation of transcripts encoding enzymes involved in photochemical reaction, Calvin cycle and photorespiration while transcripts involved in carbohydrate metabolism and utilization are up-regulated (Ainsworth *et al.,* 2008). However, the Cape Verde Island ecotype appeared to exhibit a greater capacity to acclimate to EC than the *Arabidopsis thaliana* Columbia-0 ecotype. In rice exposed to EC, transcripts encoding enzymes involved in carbon fixation such as Rubisco, carbonic anhydrase, phosphoglycerate kinase and glyceraldehyde dehydrogenase were down-regulated while transcripts encoding enzymes involved in ribulose biphosphate (RuBP) regeneration and enzymes involved in starch synthesis were up-regulated (Fukayama *et al.,* 2009). In soybean (*Glycine max*), growth under EC decreased total antioxidant capacity and response to acute oxidative stress (Gillespie *et al.,* 2011).

The combination of predicted increases in CO_2 and temperature will favour C4 grasses relative to C3 plants (Morgan *et al.,* 2011). It has been shown that a $6^{O}C$ increase of growth temperature leads to a decrease in winter wheat production by 30 % while a decrease of $6^{O}C$ in temperature increases yield by 37% (Tonkaz *et al.,* 2010). Recently, Higgins and Scheiter (2012) reported that increases in atmospheric

CO_2 associated with climate change will induce abrupt, local shifts in vegetation characterized by increased plant biomass due to the predominance of woody plant species. Their model predicts that the timing of such transitions in terrestrial ecosystems will vary globally due to variations in the rate of temperature increase and rainfall. These results suggest that global warming due to greenhouse gases will affect plants differentially, depending on their genetic characteristics as well as habitats.

Low temperature is one the most important factors which limits plant productivity. Winter hardy cereals acquire the ability to withstand cold and freezing temperature after growth and development at low temperature. This process, known as cold-acclimation (CA), is necessary for successful overwintering of winter hardy plants in the vegetative state and subsequent growth and reproduction in the following spring (Hüner *et al.,* 1993; Hüner *et al.,* 1998; Stitt and Hurry 2002; Öquist and Hüner 2003; Dahal *et al.,* 2012b). Under field as well as controlled environment conditions, maximum freezing tolerance and cold acclimation of winter cereals is attained when plants reach the 3rd to 4th leaf stage of vegetative development (Krol *et al.,* 1984; Pocock *et al.,* 2001). For this reason, the time of planting in the field in the fall is critical to ensure maximum winter survival. The effects of CA on plant morphology, physiology and biochemistry have been investigated in cold-tolerant cereals as well as spinach, *Arabidopsis thaliana*, and *Brassica napus* (Krause 1988; Hüner *et al.,* 1993, 1998; Stitt and Hurry 2002; Öquist and Hüner 2003; Savitch *et al.,* 2005; Ensminger *et al.,* 2006; Theocharis *et al.,* 2012). Cold acclimation of winter rye, winter wheat (Hüner 1985; Dahal *et al.,* 2012b), spinach (Boese and Hüner 1990) as well as *Arabidopsis thaliana* (Strand *et al.,* 1999; Gilmour *et al.,* 2000; Gorsuch *et al.,* 2010a, 2010b) and *Brassica napus* (Savitch *et al.,* 2005; Dahal *et al.,* 2012a; 2012b) generally results in a compact, dwarf growth habit with leaves that exhibit increased thickness relative to NA controls (Hüner 1985; Gorsuch *et al.,* 2010a; 2010b; Dahal *et al.,* 2012a). The increased leaf thickness associated with the cold- acclimated (CA)

state can be accounted for by either increases in leaf mesophyll cell size (Hüner *et al.,* 1981; Gorsuch *et al.,* 2010a) and/or increases in the number of palisade layers (Boese and Hüner 1990, Dahal *et al.,* 2012a). Concomitantly, these changes in phenotype for CA plants are associated with rates of CO_2 assimilation, photosynthetic electron transport and respiration that are equal to or greater than NA controls regardless of the measuring temperature as a consequence of up-regulation of photosynthetic and respiratory carbon metabolism (Hurry *et al.,* 1995; Hüner *et al.,* 1998; Stitt and Hurry 2002; Strand *et al.,* 2003; Atkin and Tjoelker 2003; Dahal *et al.,* 2012a; 2012b). Furthermore, the extent to which photosynthetic capacity is enhanced during cold acclimation is positively correlated with freezing tolerance in cereals (Öquist *et al.,* 1993; Pocock *et al.,* 2001). CA winter cereals divert metabolic energy away from vegetative growth and store the fixed carbon as an energy source for the maintenance of the CA state which is characterized by enhanced energy utilization and biomass production (Dahal *et al.,* 2012a; 2012b) coupled with increased resistance to photoinhibition and freezing tolerance and increased productivity in the spring (Hurry and Huner, 1992; Yeh *et al.,* 2000; Pocock *et al.,* 2001; Griffith and Yaish, 2004; Dahal *et al.,* 2012). These alterations in physiology and biochemistry in response to cold acclimation are mediated by differential gene expression (Sarhan and Perras, 1987; Houde *et al.,* 1992; Danyluk *et al.,* 1998; Gilmour *et al.,* 1998; Badawi *et al.,* 2008). CBF pathway appears to be important not only in regulating gene expression associated with freezing tolerance (Jaglo-Ottoson *et al.,* 1998; Benedict *et al.,* 2006; Lee and Thomashow 2012) but also in regulation of the dwarf phenotype, leaf anatomy and photosynthetic performance associated with cold acclimation (Savitch *et al.,* 2005; Dahal *et al.,* 2012a).

Although long-term exposure to EC is well documented in plants, only a few recent studies have focused on the combined effect of cold acclimation and EC where the focus was primarily on plant freezing tolerance. Bertrand *et al.,* (2007) reported

that, although exposure of *Medicago sativa* to EC stimulated plant growth, this came at a cost of reduced freezing tolerance. In contrast, Black spruce (*Picea mariana*) grown at EC under greenhouse conditions exhibited enhanced cold tolerance in the early autumn which was associated with a down-regulation of photosynthesis (Bigras and Bertrand 2006). Using a long-term, in situ CO_2 enrichment experimental design, Martin *et al.,* (2010) examined the effects of EC on the freezing tolerance of 10 alpine species. Three species were negatively affected, 2 species marginally affected and 5 species exhibited no significant effects of EC on freezing tolerance.

With the predictions of increased carbon dioxide concentration associated with global warming, we hypothesized that CA winter cultivars will maintain a photosynthetic capacity equal to or greater than NA controls under long-term growth and development at EC under controlled environment conditions. Although the differential phenotypic plasticity of CA and NA cereals was investigated in ambient CO_2 (Dahal *et al.,* 2012) and in *Brassica napus* under ambient as well as EC (Dahal *et al.,* 2012a), the effects of long-term exposure to EC on global gene expression in NA and CA wheat or *Brassica napus* has not been addressed. Using wheat Affymetrix microarrays, we compared the transcriptome of CA and NA wheat leaves under long-term growth and development at either ambient or EC. We report that growth of NA wheat under EC conditions resulted in the down-regulation of genes associated to biotic stress responses. In contrast, not only did CA wheat maintain enhanced photosynthetic energy use efficiency but cold acclimation almost completely reversed the down regulation of biotic stress-related genes during growth at EC.

4.4 RESULTS

4.4.1 Effects of elevated CO2 on growth characteristics

To assess potential pot size limitations, NA and CA plants were grown to maturity at either ambient or EC in pots of varying volume (0.5, 2.0, 4.0 and 6.0L). Under ambient CO_2, growth conditions under either NA or CA conditions, we detected no significant effect of pot size on total dry matter accumulated (data not shown). However, under EC conditions, growth of plants in pot volumes of 2.0L or less caused a 30% decrease in total plant biomass accumulation relative to growth in either 4.0 or 6.0L pots (data not shown). These results are consistent with those recently reported for the effects of pot size on *Brassica napus* grown at either ambient or EC (Dahal *et al.,* 2012a). Thus, for consistency, NA and CA Norstar were grown in 4L pots at either ambient or EC to ensure no measurable sink limitations due to pot size.

CA Norstar exhibited a compact, dwarf growth habit and altered leaf morphology relative to NA counterparts at ambient CO_2 (Fig. 1). The third leaves of all NA plants were fully expanded between 20-25 days and those of CA cultivars between 70-80 days (data not shown) irrespective of CO_2 conditions. This is due to the fact that the exponential growth rate of CA Norstar was about 33% of that observed for NA Norstar (Table 1) and is consistent with previous published reports for the comparison of NA and CA wheat and rye cultivars (Hüner *et al.,* 1993; Hurry *et al.,* 1992; Pocock *et al.,* 2001; Dahal *et al.,* 2012b). Growth of Norstar under EC had minimal effects on exponential growth rates at either 20° or 5°C (Table 1).

EC significantly increased shoot dry matter accumulation by about 30% in NA and CA Norstar (Table 1; Fig. S1A, closed versus open circles). It appears that the increase in shoot dry matter accumulation at elevated versus ambient CO_2 was

primarily associated with increased plant size (Fig. 1). EC significantly increased Chl per unit leaf area in NA Norstar (Table 1). Although cold acclimation of Norstar winter wheat at ambient CO_2 increased specific leaf weight (SLW, g dry weight m^{-2} leaf area) by about 2.4-fold, Chl per unit leaf area by 30%, and leaf protein content 2.5-fold relative to NA Norstar (Table 1), growth at EC had minimal effects on these parameters in CA Norstar. Although 75 day old CA plants exhibited a dwarf phenotype compared to 25 day old NA Norstar (Fig. 1), CA plants exhibited similar total shoot biomass as NA plants (Table 1) which could be accounted for by the 2.4-fold increase in specific leaf weight (Table 1). This biomass was accommodated by increased leaf thickness in CA compared to NA Norstar which is consistent with previous results for winter rye (Hüner 1985), spinach (Boese and Hüner 1990), *Arabidopsis thaliana* (Strand *et al.,* 1999; Gorsuch *et al.,* 2010a) and *Brassica napus* (Dahal *et al.,* 2012a).

4.4.2 Effects of elevated CO2 on photosynthesis and respiration

Results in Fig. 2 illustrate the light response curves (Fig. 2A, 2B) and light-saturated CO_2 response curves (Fig. 2C, 2D) of gross CO_2 assimilation for fully developed third leaves of Norstar winter wheat measured at 20°C. NA (Fig. 2A, open circles) and CA (Fig. 2B, open circles) exhibited comparable light response curves for A_{sat}. However, growth under EC stimulated light saturated A_{sat} by about 50% in both NA and CA Norstar relative to ambient CO_2 (Fig. 2A, B, closed versus open circles). The CO_2 response curves for A_{sat} were comparable for both NA (Fig. 2C) and CA (Fig. 2D) grown under either ambient (open circles) or EC (closed circles). Thus, growth under EC had minimal effects on the CO_2 response curves for both NA (Fig. 2C) and CA (Fig. 2D) Norstar.

Excitation pressure, estimated as 1-qP, is a measure of the relative redox state of Q_A, the first, stable quinone electron bound to the reaction centres of PSII and

provides an estimate of the proportion of closed PSII reaction centres (Hüner *et al.,* 1998; Ensminger *et al.,* 2006). The light response curves for NA Norstar grown at EC (Fig. 3A, closed symbols) indicates that excitation pressure tends to be higher for a given irradiance than Norstar grown at ambient CO_2 (Fig. 3A, open symbols). The inverse of the maximum initial slope of the light response curves is an estimate of the quantum requirement for PSII closure (Rosso *et al.,* 2009; Dahal *et al.,* 2012a), that is, the number of photons required to close PSII reaction centers. The data in Fig. 3A indicate that the quantum requirement to close 50% of PSII reaction centers in NA Norstar grown under EC (800 photons) was about 33% lower than that for NA Norstar (1200 photons) grown at ambient CO_2. In contrast, the light response curves for excitation pressure for CA Norstar (Fig. 3B) indicated minimal differences in 1-qP at a given irradiance for plants grown at either elevated (Fig. 3B, closed symbols) or ambient CO_2 levels (Fig. 3B, open symbols). Furthermore, the quantum requirement to close 50% of PSII reaction centers in CA Norstar (1744 photons) was 1.45-fold and 2.2-fold greater than NA Norstar grown at either ambient or EC respectively.

Since CO_2 is the major electron acceptor in photosynthetic carbon assimilation, increasing the concentration of CO_2 should result in a decrease in excitation pressure measured as 1-qP. The results of Fig. 3C and 3D illustrate the CO_2 response curves measured under light saturating conditions for NA (Fig. 3C) and CA Norstar (Fig. 3D). As expected, increasing CO_2 concentrations caused a comparable decrease in excitation pressure irrespective of whether NA and CA Norstar were grown at either ambient (open symbols) or EC (closed symbols). However, the CO_2-saturated excitation pressure for CA Norstar (1-qP = 0.310) (Fig. 3D) was about 45% lower than that of NA Norstar (1-qP = 0.480) (Fig. 3C).

To protect photoautotrophs from potential damage due to the absorption of excess light, photosynthetic organisms have evolved a photoprotective mechanism that dissipates a proportion of the excess light not utilized in photosynthesis as heat. This photoprotective process is called nonphotochemical quenching (NPQ) (Demmig-Adams and Adams 1992; Ort 2001; Li *et al.,* 2009). The results illustrated in Fig. 4 indicate that growth of NA Norstar at EC decreased the quantum requirement per unit of NPQ (563 photons per unit of NPQ) by about 60% compared to that for NA Norstar grown at ambient CO_2 (900 photons per unit of NPQ) as estimated by the calculating the inverse of initial slopes of the light response curves for NPQ (Fig. 4A). In contrast, growth of CA wheat at EC appeared to have a smaller effect and decreased the quantum requirement for NPQ in CA Norstar (889 photons per unit NPQ) by only about 30% relative to that observed under ambient CO_2 (1189 photons per unit NPQ) (Fig. 4B). However, the quantum requirement for CA Norstar was higher than that of NA Norstar regardless of the growth [CO_2]. As expected, increased [CO_2] caused a decrease in NPQ in NA and CA plants grown either at ambient or EC (Fig. 4C and D). However, the NPQ observed under saturated CO_2 was 40-50% lower in CA than NA.

The results of Table 1 indicate that CA Norstar exhibited rates of respiration that were about 26% higher than NA Norstar when plants were grown at either ambient or EC, which is consistent with published reports (Atkin and Tjoelker 2003). Thus, rates of respiration for Norstar winter wheat appeared to be sensitive to growth temperature but not growth CO_2 levels (Table 1).

4.4.3 Effects of cold-acclimation and elevated CO2 on stomatal characteristics

Growth at EC suppressed stomatal conductance by 35% in NA Norstar and, as a consequence, leaf transpiration rates decreased by 30% and WUE increased by 125% in NA Norstar upon growth at EC compared to ambient CO_2 (Table 1). In

contrast, EC induced no change in stomatal conductance, as well as leaf transpiration rates but enhanced the WUE by 85% in CA Norstar compared to ambient CO_2 (Table 1). This was due to the fact that cold acclimation had already suppressed stomatal conductance by about 40% at ambient CO_2 relative to NA Norstar (Table 1).

It appears that cold acclimation and EC-induced suppression of gs can be accounted for, in part, by a decrease in stomatal density on both abaxial and adaxial leaf surfaces in response to growth at EC (Table 1). These data indicate that the enhancement of WUE induced by cold acclimation and EC is primarily associated with an increase in the light-saturated CO_2 assimilation and decrease in stomatal conductance upon cold acclimation or growth at EC.

4.4.4 Identification of genes regulated by elevated carbon dioxide in non-acclimated wheat

A total of 1022 genes representing 1,6 % of all genes on the microarray were differentially regulated by at least two-fold between ambient (AC) and EC in NA wheat (Fig. S2A). The proportion of down-regulated (768) genes was 3 times higher than the up-regulated ones (254). Furthermore, the down-regulated genes were more affected (20 genes with more than 10 fold change) than the up-regulated ones (only 1 gene with more than 10 fold change) (Table S2).

Among the 1022 differentially regulated genes, 355 were classified in known functional categories. Table 2 shows that EC affects several gene categories. A large proportion of the regulated genes are those involved in stress responses (biotic: 43 and abiotic: 24). Several genes involved in different aspects of cellular metabolism are also affected including primary metabolism (carbohydrate: 40, amino-acid: 11, and lipid metabolism 11); secondary metabolism (20), and hormone metabolism (17).

Genes involved in regulation of different processes such as transcription (40), protein post-translational regulation (15) or degradation (9) and signal transduction (11) are also abundant. Many genes involved in oxidation-reduction (23) and transport (25) are regulated. Interestingly, genes affecting organelle metabolism are also involved (chloroplast/photosynthesis: 9 and mitochondrial transport: 2). The detailed description and fold change of the different categories of genes are listed in Table S3.

To highlight pathways and biological functions that are co-ordinately regulated by EC under NA condition, we used the MAPMAN software. An overview of genes co-ordinately regulated by EC and involved in abiotic and biotic stress responses is presented in Fig. 5. The genes and their fold change are listed in Table S4. Most of these genes were down-regulated and encode regulatory proteins (transcription factors: WRKY, ERF and MYB), signal transducing molecule related to sugar, nutrient and physiology (pyruvate dehydrogenase kinase) light and calcium signalling, receptor kinase (DUF, LRK10-like, leucine-rich and wall associated kinase), protein G and MAPK and hormone (abscissic acid, jasmonate) that trigger the molecular response of biotic and abiotic stresses. MAPMAN was combined with the Benjamini Hochberg statistical analysis to cluster and compare the number of regulated genes within a cellular category/functional group/pathway to the total number of genes annotated in this functional group (Bischof *et al.,* 2011). According to Andersson *et al.,* (2004) the mean value of the fold change of genes involved in a particular category is a good approximation in the relative effort plant makes to synthesize protein of the respective categories. Clustering of the genes according to their fold change similarity identified several functional categories of genes. Among all the functional groups identified, those exhibiting a statistically significant difference are listed and shown in Table 3 and Fig. 6. The functional group containing the highest number of regulated genes are respectively those involved in

stress responses (biotic and abiotic), the major Carbohydrate (CHO) metabolism, RNA regulation of transcription through ARR transcription factor, RNA-RNA binding and hormone metabolism.

Besides the functional groups of genes identified with co-ordinated expression pattern and by clustering according to fold change similarity, the transcripts that show an important regulation were analysed. The most down-regulated genes (between -90 and -9 fold change) correspond to thionin-like proteins (three entries: -90.2; -58.0; and -51.1 fold) related to biotic stress, a thiol protease (-33.4 fold) protein related to protein degradation, two putative plantacyanin containing domain protein (Os03g0709300 -21.8; and -15.9 fold) potentially involved in reproduction, a dehydrin (-20.2 fold) involved in abiotic stress, a myb transcription factor (-20.0) related to RNA regulation of transcription, a putative jasmonate induced protein (Os12g0198700: -15.1 fold) two bowman Birk protease inhibitors (-10.9 and -9.7 fold), a linalool synthase (-10.6 fold) related to secondary metabolism, cytochrome oxidase C (-9.8 fold) of mitochondria electron transport and 6 unknown genes (Ta.15129.1.S1_at: -14.1 fold; Ta.521.1.S1_at: -14.1 fold; Ta.22572.1.S1_at: -13.6 fold; Ta.521.1.S1_s_at: -10.7 fold; Ta.13300.1.S1_at: -10.1 fold; Ta.23327.1.S1_at: -9.02 fold; Table S2).

The most highly up-regulated genes (between 15 and 4.9 fold change) correspond to: a hypothetical protein containing a domain similar to the pumilio-RNA binding protein (Os12g0493900 : 15.8 fold) in *Oryza sativa* that can regulate the activity of various RNA molecules; a protein with high similarity (81%) to the Al malate transporter ALMT3 (P0657H12.23: 10.0, 9.4 and 8.8 fold); a member of the aquaporin family (8.6 fold); members of the germin-like proteins (8.1, 6.6, and 4.9 fold); the metal ion transporter Zip3 (7.2 fold); the cold responsive protein WCOR14 targeted to the chloroplast; and unknown genes (Ta.22063.1.S1_S_at: 9.5 fold;

Ta.104641.1.S1_at: 7.8 fold; TaAffx.6284.1.S1_at: 6.7 fold; Ta.28468.2.S1_s_at: 6.1 fold; Ta.28468.1.A1_at: 5.6 fold; Table S2).

Since photosynthetic down-regulation was associated with long-term acclimation during exposure to EC in certain species (Drake *et al.*, 1997; Ainsworth and Long, 2005) we examined whether other genes involved in photosynthesis are regulated by more than 2-fold between AC and EC. Several genes including ATP synthase, NADH dehydrogenase, photosystem I P700 chlolorophyll a apoprotein, photosystem II 10 kDa polypeptide, and phosphoribulokinase were up-regulated (Table S3).

However, by far, the most striking finding is that many of the genes down-regulated by EC in NA wheat are related to the plant defense system. Overall, 121 genes that are down-regulated by EC are known to be associated with pathogen and defense mechanisms (Table 4).

4.4.5 Identification of genes regulated by elevated CO2 in cold-acclimated wheat

A total of 372 genes representing 0,6 % of the genes on the microarray was differentially regulated (Fig. S2b). Most of these genes were down-regulated (253) but only 7 had a fold change lower than -5 with the lowest fold change at -13. On the other hand, for the up-regulated genes (118) the highest fold change was 9 (Table S5).

Among the 372 differentially regulated genes, 255 with known function were mapped in functional gene categories (Table 5). The fold change and gene description are listed in Table S6. As found with NA plants, a large proportion of the regulated genes were related to abiotic (19) and biotic (3) stresses in CA plants. However, in CA plants, only 3 genes were associated with biotic stress (Table 5) while there were 43 in NA plants (Table 2). Several aspects of cellular metabolism were also affected

including primary carbohydrate metabolism (9) and secondary metabolism (9). Genes involved in transcription are also abundant (14). Many genes were involved in redox biochemistry (16), transport (7) and hormone metabolism (4). Interestingly, genes involved in photosynthesis (7) are well represented. Furthermore, genes involved in the protection against photooxidative damage in the chloroplast (ELIP) are found in CA plants contrary to NA plants. The functional classification and fold change of the different genes are listed in Table S6. As with NA plants, we also examined more closely other genes involved in photosynthesis. Genes encoding for ATP synthase subunit alpha (-5.2; -4.9 and -3.1 fold) and photosystem I P700 chlorophyll a apoprotein (-3.4 and –2.7 fold) were down-regulated while one gene encoding for cytochrome b559 subunit alpha was up-regulated by 2.5 fold. The two down-regulated genes responded in the opposite direction compared to NA plants indicating that excess energy under low temperature is an important factor to be considered under EC. In carbohydrate metabolism, hexokinase-7 (-2.8 fold), extracellular invertase (-2 fold), beta-glucanase (-3.5; -3.4; -2.6 fold), beta-D-glucan exohydrolase were down-regulated.

We also used clustering with MAPMAN software and the Benjamini Hochberg corrected wilcoxson rank sum test to identify categories of genes that have a statistically different behaviour in terms of fold change similarity compared to all the categories identified in Table 5. Three categories corresponding to the protection against photooxidative damage in the chloroplast, involving ELIP, secondary metabolism and protein folding were retained by this test and represent functional categories or pathways that may be co-ordinately regulated by EC in CA plants (Fig. 7). The name and fold change of the corresponding genes are listed in Table 6.

Other transcripts that show an important differential expression were also examined (Table S5). The most down-regulated genes encode two senescence-

associated proteins related to development (-13.1 and –9.7 fold). Several other genes of potential interest include a superoxide dismutase involved in oxidative stress (-5.7 fold), ATP synthase (-5.2 and –4.9 fold) involved in photosynthesis, a hypothetical protein related to heat shock protein DnaJ (Os05g0529700: -5.6 fold); a bacterial peptide synthetase (-5.1 fold), a hypothetical protein with a protein-kinase like domain (Os10g0153900: 4.6 fold); a bacterial filamentous hemagglutinin/adhesin (-4.4 fold), a member of the arabinogalactan protein family (Os03g0188500: -4.4 fold); an oxalate oxidase related to abiotic stress (-4.3 and –4,2 fold), and a histidine-rich glycoprotein (-4.1 fold). The remaining genes among the 20 most down-regulated encode unknown proteins (TaAffx.21593.1.S1_at: -7.2; Ta.17978.1.S1_at: -4.8; TaAffx.54099.1.S1_s_at: -4.6; Os03g0188500: -4.3; Sb07g000490: -4.28; Os05g0184901: 4.21 and 4.21 fold; Table S5).

The most up-regulated genes could be classified in the following way: six genes involved in secondary metabolism (two dihydroflavonol 4-reductases: 9 and 4.8 fold; one flavonone 3-hydroxylase: 8.2 fold; three chalcone synthase: 5.1, 4.5 and 4.4 fold); six genes encode for high or low molecular weights ELIP proteins involved in the protection against photooxidative damage in the chloroplast (7.0; 6.9; 6.4; 6.0; 4.9 and 4.4 fold); two genes encode enzymes involved in protein degradation: one with 91% similarity to a serine type endopeptidase (7.2 fold) and another with 82% similarity to *Zea mays* glutamate carboxypeptidase (4.5 fold); one gene similar to Os07g0631900 encode a putative 29 kDa ribonucleoprotein A of the chloroplast (4.6 fold). The last 5 entries are unknown genes (Ta.20215.1.S1_at: 6.1; Ta.23419.3.S1_x_at: 5.5; Os01g0143800: 4.8; Ta.15028.1.S1_at: 4.7 fold: Table S5).

4.4.6 Comparison of non-acclimated and cold-acclimated wheat transcriptome under elevated CO2

In order to identify the EC-responsive genes that are common in NA and CA plants, Venn diagram analysis were performed with the genes differentially regulated between ambient and EC in NA plants (1022 genes) and those regulated in CA plants (372 genes). There are 77 common regulated genes by EC in both NA and CA wheat. A total of 945 genes were specifically regulated by EC in NA while 295 were specifically regulated by EC in CA wheat (Figure 8A).

The 77 common EC-responsive genes in NA and CA wheat are shown in Table S7. Among these, a total of 9 genes showed opposite regulation (highlighted in red in Table S7: i.e. up-regulated in NA and down-regulated in CA or vice versa). The remaining 69 genes have the same expression pattern in both conditions. Besides the functional category shared by both NA and CA, there were genes specific to each set of plants (NA and CA plants). For example some genes involved in protein synthesis and folding were up-regulated by EC in CA plants but not in NA plants (Figure 8 B). Furthermore, we compared the genes regulated by CA at ambient CO_2 and the genes regulated by EC in NA plants. We identified 241 genes down-regulated by EC in NA plants but up-regulated by CA at ambient CO_2 (Table S8). Almost half of the pathogen related genes down-regulated by EC in NA plants (Table 4) are up-regulated during cold acclimation at ambient CO_2 (Table S9). These include 48 genes related to cell wall, proteolysis, beta glucanase, signaling, secondary metabolites and gluthatione-S-transferase (Fig. 9 and Table 7). Furthermore, 61 genes were up-regulated by EC in NA plants but down-regulated by CA at ambient CO_2. Finally 253 genes were down-regulated by both EC and CA (Table S8). This last observation is quite significant regarding the fact that the two most down-regulated genes by CA (lines 242-243 in Table S8 and lines 4-5 in Table S9) correspond to the same sulfur-rich thionins protein that are the most repressed by EC (Table S2).

4.4.7 Gene validation of the microarray by quantitative real-time PCR

To validate the microarray data, quantitative real-time PCR was performed with selected genes from the array. Genes involved in defense response, cold acclimation and photosynthesis were analysed. Pathogen-related genes (PR1B; chitinase and thaumatin-like protein) were down-regulated by EC in NA plants and up-regulated by cold acclimation at ambient CO_2 (Table 8). The expression of genes involved in photosynthesis like ribulose 1,5-biphosphate carboxylase/oxygenase (rbcl), photosystem Q(B) PsbA, sucrose phosphate synthase 1 (SPS1) and chloroplastic fructose biphosphatase (cFBPAse) did not change with EC or cold treatment except for PsbA that was slightly up-regulated by EC. Genes known to be induced by cold acclimation, like the WCOR518 and ice recrystalization inhibition protein (TaIRI) were also tested and showed up-regulation by cold treatment. The WCOR518 gene was also up-regulated by EC. The correlation coefficient between the qRT-PCR and microarray data is 0.7919 demonstrating that expression levels quantified by real-time PCR gave similar results to those found by microarray (Fig. S3, Table 8).

4.5 DISCUSSION

A confounding problem in research on plant cold acclimation is to distinguish between responses at either the molecular, biochemical or physiological levels associated with cold stress versus those associated with cold acclimation. Exposure to cold stress can be imposed simply by rapidly shifting control NA plants grown at warm temperatures (eg. 25°C) to low temperature (eg. 5°C) for a short, given period of time. In contrast, cold acclimation requires growth and development at low temperature to establish a new homeostatic state which requires a long-term exposure to low temperature (Hüner *et al.,* 1993; Hurry *et al.,* 1994; Gray *et al.,* 1997; Atkin and Tjoelker 2003; Armstrong *et al.,* 2006; Gorsuch *et al.,* 2010a; 2010b; Dahal *et al.,* 2012a; 2012b). Because growth at low temperature is thermodynamically constrained to a greater extent than growth at warm temperatures, chronological age can not be used to compare NA versus CA plants. Comparative growth kinetics based on a combination of shoot dry weight accumulation, leaf area as well as leaf number are critical to establish comparable developmental states during growth under control versus cold acclimation conditions. Thus, based on comparative growth kinetics and leaf number, we established that NA Norstar grown at 20°C for 25-30 days were at a comparable development state to CA Norstar grown at 5°C for 70-80 days. This is consistent with previous results reported for winter rye (Krol *et al.,* 1984; Gray et al, 1997), various winter wheat cultivars (Hurry *et al.,* 1994) as well as *Arabidopsis thaliana* (Rosso *et al.,* 2009). We used a comparable approach in this study to ensure that any differences detected in phenotype, physiology, and gene expression were due to either CA or EC and not due to differences in developmental states. The importance of such an experimental approach in the study of cold acclimation has been emphasized previously (Krol *et al.,* 1984; Gray *et al.,* 1997; Atkin and Tjoelker 2003; Gorsuch *et al.,* 2010a; Dahal *et al.,* 2012b).

4.5.1 Elevated CO2 differentially down-regulates defense genes associated with biotic stress

EC affects different gene categories as shown in Table 2. The most represented genes are involved in abiotic/biotic stresses, carbohydrate metabolism, RNA regulation of transcription, oxidation-reduction process, transport, secondary and hormone metabolism. The down-regulation of transcript related to defense is one of the most striking effects of EC on NA wheat plants. At least 121 genes are pathogen related (PR) or systemic acquired resistance (SAR) transcripts demonstrating a major impact on defense-related transcripts (Table 4). To invade the plants, pathogens must overcome the different protection mechanisms involving the cell wall, secondary metabolites and antimicrobial proteins. Several transcripts involved in cell wall metabolism and secondary metabolism were down-regulated by EC (Fig. 5 and Table S4). Pathogen-elicitors are sensed by plants and induce a reinforcement of cell walls, production of phytoalexins and the biosynthesis of defense-related proteins. One of the most prominent defense mechanism of plants upon biotic stress exposure is the induction of PR proteins and the products of the SAR response. To date at least 17 family of PR proteins were identified (Van Loon *et al.,* 2006). These PR proteins possess either antimicrobial activities through hydrolytic activities on cell walls, or contact toxicity and sometimes they can act in defense signaling (Van Loon *et al.,* 2006). Plant chitinase II and IV, WIR protein, and thaumatin-like proteins (Table 4) are well known for their defense role against pathogen attack (Schlumbaum *et al.,* 1986; Mauch *et al.,* 1988; Vigers *et al.,* 1992; Muthukrishnan *et al.,* 2001). The down-regulation of these pathogen related transcripts (Table 4) in response to EC suggests that NA wheat grown at EC is more vulnerable to pathogen attack.

The induction of PR proteins is mediated by the action of the signaling compounds salicylic acid, jasmonic acid, or ethylene (Thomma *et al.,* 2001). In our

arrays, the transcript of 1-aminocyclopropane-1-carboxylate oxydase and lipoxygenase (LOX) involved in ethylene or jasmonic acid signaling during pathogen attack were also down-regulated similar to the data revealed by the transcriptional profiling between AC and EC in soybean (Casteel *et al.,* 2008). It has been shown that resistance of soybean to Japanese Beetles (*Popillia japonica*) was lowered as key transcripts associated with defense and hormone signaling were down-regulated under EC (Casteel *et al.,* 2008).

Several protein kinases and phosphatases play crucial roles in signaling during pathogen recognition and the subsequent activation of plant defense mechanisms (Romeis, 2001). We identified several transcripts encoding protein kinases that are down-regulated in NA wheat exposed to EC. These genes encode for CIPK-like protein, Calcineurin B-like interacting protein kinase, serine threonine-protein kinase and phosphatase, abscissic acid-inducible protein kinase, probable protein phosphatase 2C (Fig. 5 and Table 4). These transcripts encoding signaling factors were up-regulated in the interaction between wheat and stripe rust fungus *Puccinnia striiformis* f. sp. tritici (*Pst*) and are believed to be involved in the defense resistance of wheat against *Pst* (Wang *et al.,* 2010). However it is difficult to assign induced or decreased changes of gene expression to successful defense or penetration against pathogen (Gjetting *et al.,* 2007). Like metazoans, plants have an innate basic non-specific and pathogen race-specific immunity (Bischof *et al.,* 2011). It has been demonstrated that plant recognize pathogen-associated molecular patterns (PAMPs) through pathogen receptors (PRRs) localized on their plasma membrane (Albert *et al.,* 2010). These PRRs can lead to a cascade of mitogen activated protein kinase (MAPK) and the subsequent activation of WRKY transcription factors (TFs). WRKY TFs are involved in the immunity that plants develop after a previous exposure to a pathogen as revealed in some studies showing the support or limitation of basal resistance against pathogen by WRKY (Asai *et al.,* 2002; Eckey *et al.,* 2004; Shen *et al.,* 2007).

In our array experiment, we have identified a WRKY45 transcription factor that is down-regulated. This WRKY45 is similar to two allelic rice WRKY45-1 and WRKY45-2 that have opposite roles in rice resistance against certain bacterial pathogens but could play positive roles against other pathogens due to the activation of different gene subsets (Tao *et al.,* 2009). The down-regulation of WRKY45 in wheat suggests that NA wheat grown under EC may have a lower capacity to stimulate the WRKY pathway and consequently pathogen invasion may be easier under EC. The fact that the 3 most down-regulated genes with a fold change of -90, -58, and -53 encode thionins proteins that were previously found to be induced in wheat by benzothiadiazole, a compound known for its activation of SAR and WRKY45 in plants (Gorlach *et al.,* 1996) also suggests that pathogen resistance is reduced under EC. Thionins are a group of small (≈5000 da) sulfur-rich proteins found in leaves and stems that are secreted into vacuoles, protein bodies and cell wall. They have antimicrobial, antifungal and antiviral properties (Benko-Iseppon *et al.,* 2010).

Transcripts involved in secondary metabolism were down-regulated in NA but up-regulated in CA wheat during long term exposure to EC as shown in Tables S3 and S6. The transcription factor that displayed the highest fold change correspond to a MYB transcription factor (-20 fold) similar to the Myb-related protein Hv1 present in germinating seeds and in shoot and root apical meristems of *Hordeum vulgare* involved in the response to external stimuli (Wissenbach *et al.,* 1993). This transcription factor is believed to be involved in the transcription of genes for the biosynthesis of flavonoids (Marocco *et al.,* 1989). Lignan and lignin are plant cell wall components, which play a role as structural strengthening elements, but also constitute a primary defense barrier against pathogens. The other remaining secondary metabolism genes identified participate in the synthesis of flavonols, alkaloid-like, tocopherol, betaine and carotenoids. Theses classes of secondary

metabolites are well known for their defense properties against pathogen, herbivory and reactive oxygen species (Stafford and Ibrahim, 1992; Zhou *et al.,* 2006).

A comparison of long-term FACE experiments versus controlled environment chamber CO_2 experiments has been recently reviewed (Ainsworth *et al.,* 2008). Results of experiments conducted on long-term exposures to EC in controlled environment chambers indicate that plant growth at EC levels predicted for 2050 stimulates leaf CO_2 assimilation between 20-40% (Bernacchi *et al.,* 2006). The results of FACE experiments with *Glycine max* L. indicates that exposure to EC for 3 consecutive growing seasons resulted in smaller enhancement of CO_2 assimilation rates than previously predicted (Bernacchi *et al.,* 2006). Furthermore, Zavala *et al.,* (2008) reported that growth of soybean at EC increased susceptibility to beetle pests as indicated by a down regulation of genes involved in defence signalling. Ainsworth *et al.,* (2008) suggested that a limitation of chamber CO_2 experiments is their inability to predict complex abiotic and biotic interactions as a consequence of long term growth of plants under EC. Contrary to this conclusion, we provide evidence that carefully designed chamber experiments can indeed predict complex biotic and abiotic interactions at the gene expression level associated with plant growth under EC. In contrast to NA wheat, CA induced most transcripts involved in secondary metabolism during long term exposure to EC. (Fig. 7 and Table S6) suggesting that CA plants during EC have more potential to resist to pathogen invasion and UV-stress. Among these, chalcone synthase and dihydroflavonol-4-reductase are up-regulated transcripts in CA wheat exposed to EC and represent key genes in phytoalexins and anthocyanins synthesis (Ferrer *et al.,* 1999).

Down-regulation of transcripts related to the biosynthesis of these defense-related genes indicates that NA Norstar grown at EC may be very susceptible to

invasion by pathogens, herbivores and photo-oxidative stress compared to their CA counterparts. However the 2 most down-regulated genes by EC in CA plants (-13.1 and –9.7 fold) are involved in senescence suggesting that it will be delayed in CA plants under EC. The programmed cell death associated with senescence can be accelerated by environmental factors such as shading, temperature and pathogens. Senescence acceleration can decrease the probability of pathogen attack and its delay may increase pathogen sensitivity. Several senescence associated genes encode PR proteins involved in the SAR of plants (Quirino *et al.,* 2000; Van Loon *et al.,* 2006). However, delaying senescence can prevent chloroplast breakdown ensuring the production of photosynthate for a longer period (Bùchanan-Wollaston and Ainsworth, 1997).

CA wheat is less affected by EC as revealed by the lower number of genes regulated compared to NA wheat. This observation can be explained by the fact that cold acclimation has regulated most of the genes that change during growth at EC in the opposite direction. Winter hardy cereals like wheat and rye are known for their wide tolerance to abiotic stresses. Changes in the expression of a large number of transcripts upon exposure to cold have long been known. We have previously identified several low-temperature induced genes in wheat having regulatory, signaling or protective roles against cold and freezing temperature (Houde *et al.,* 1992; NDong *et al.,* 2003; Badawi *et al.,* 2007; Badawi *et al.,* 2008). CA plants go through physiological and biochemical events that help to prevent damage associated with cold and freezing temperatures. Several transcripts associated to defense against abiotic and biotic stress like chitinases, thaumatin, ß,1,3-glucanase, are up-regulated during cold acclimation (Danyluk *et al.,* 1998; Yeh *et al.,* 2000; Tremblay *et al.,* 2005; Kuwabara and Imai, 2009). The up-regulation of these transcripts was confirmed in the current study (Table S9). The most up-regulated genes encode defense-related

genes like phenylalanine ammonia-lyase (129 fold); ent-kaurene oxidase 1 (110 fold); beta-1,3-glucanase (105 fold); chalcone synthase (86 Fold).

Even though CA strongly induced defense related transcripts, the gene that is most down-regulated by cold (sulfur-rich protein/thionin; -264; -201 fold) is associated to SAR suggesting that cold acclimation doesn't fully confer resistance against pathogens. Genes involved in SAR were strongly down-regulated in both NA and CA wheat exposed to EC, as well as in CA wheat at ambient CO_2.

4.5.2 CA Norstar maintains photosynthetic performance during long-term exposure to EC

Although CA of Norstar winter wheat induced a dwarf phenotype (Fig. 1) and reduced growth rates by about 30% (Table 1) relative to NA Norstar, CA Norstar (Fig. 2B) maintained comparable photosynthetic efficiency, as measured by the initial maximum slope of the light response curve (Fig 2B), as well as comparable light saturated rates of photosynthesis (Fig. 2D) as NA Norstar (Fig. 2A and 2C) when grown either under ambient or EC conditions. These results for ambient CO_2 conditions are consistent with previous reports that CA winter cereals either maintain comparable rates or exhibit higher light saturated rates of photosynthesis than their NA counterparts (Hüner *et al.,* 1993; Dahal *et al.,* 2012b). Here we report that CA Norstar maintains comparable rates to NA Norstar even after long-term growth at EC. Furthermore, growth at EC stimulated light saturated rates of photosynthesis to a similar extent (Fig. 2A and 2B) and the CO_2 response curves for A_{sat} were similar for NA and CA Norstar irrespective of levels of CO_2 experienced during growth. This contrasts with the recent report for *Brassica napus* which showed that growth of NA *Brassica* at EC significantly inhibited light saturated A_{sat} relative to CA *Brassica* (Dahal *et al.,* 2012a). These results were interpreted to indicate that NA but not CA *Brassica napus* is susceptible to feedback limited photosynthesis when grown at EC.

Thus, this phenomenon appears to be species dependent since neither NA nor CA Norstar winter wheat exhibited any significant inhibition of A_{sat} when grown under EC conditions (Fig. 2).

The data illustrated in Fig. 2 indicate that CA Norstar is photosynthetically acclimated to growth at low temperature. CA Norstar maintains significantly higher rates of light saturated CO_2 assimilation which are coupled to significantly higher rates of photosynthetic electron transport than NA Norstar at all measuring temperatures below 25°C (Dahal *et al.,* 2012b). In addition, Dahal et al (2012b) reported that this was associated with increased levels of Rubisco, cFBPase, psbA and psaA upon cold acclimation. Consequently, CA Norstar exhibited a higher quantum requirement to close PSII reaction centers than NA Norstar irrespective of $[CO_2]$ during growth (Fig. 4A and 4B). This reflects an increased capacity to keep PSII reaction centers open by increased consumption of photosynthetic reductants. Consistent with this interpretation is our observation that CA Norstar also exhibits lower excitation pressure, that is, a greater proportion of open reaction centers, under CO_2 saturated conditions than do the NA plants (Fig. 3C and 3D). These results are due, in part, to the increased photosynthetic apparatus per unit leaf area in CA versus NA Norstar as indicated by an approximate doubling in specific weight and total leaf protein per leaf area as well as a 1.3-fold increase in Chl per leaf area (Table 1). These results are consistent with previous reports for cold acclimation of winter cereals (Hüner *et al.,* 1993; Dahal *et al.,* 2012b), *Brassica napus* (Savitch *et al.,* 2005; Dahal *et al.,* 2012a) as well as *Arabidopsis thaliana* (Savitch *et al.,* 2001). Furthermore, growth under EC conditions does not suppress these photosynthetic characteristics associated with CA Norstar.

It is well established that enhanced CO_2 availability for Rubisco leads to more carbon fixation (Ainsworth and Long, 2005). Our microarray analyses show that

phosphoribulokinase is up-regulated in CO_2-enriched wheat leaves. This enzyme catalyzes the formation of RuBP. These results support the hypothesis that plants respond to high carbon dioxide exposure by modulating the regeneration of the Rubisco substrate RuBP (Chen *et al.,* 2005; Wang, 2009). Transcripts encoding polypeptides of the photosynthetic electron transport chain are up-regulated (Table S3) which is consistent with the increased rates of light saturated CO_2 assimilation (Fig. 2) as well as increased biomass (Fig. S1) and increased specific leaf weight (Table 1). In contrast to other published reports which indicate a possible down-regulation of Rubisco or a limitation of RuBP regeneration during long-term exposure to high CO_2 (Drake *et al.,* 1997), we detected no inhibition of light saturated or CO_2 saturated rates of photosynthesis in either NA or CA Norstar winter wheat upon growth and development at EC. However, we did detect a decrease in the quantum requirement for the closure of PSII reaction centers in NA Norstar grown at EC relative to plants grown at ambient CO_2 (Fig. 3A). This was coupled with a decrease in the quantum requirement for NPQ (Fig. 4A). In contrast, growth of CA Norstar at EC had little effect of the quantum requirement to close PSII reaction centers which was coupled with a smaller effect of EC on the quantum requirement for NPQ. This implies that CA Norstar is able to maintain higher energy use efficiency than NA wheat regardless of CO_2 conditions during growth. However, there was no effect of feedback limited photosynthesis evident from either the light or CO_2 response curves for CO_2 assimilation (Fig. 2). In carbohydrate metabolism, several genes coding for enzymes involved in sucrose degradation were down-regulated by EC in NA wheat. These include beta-amylase, fructokinase, fructan 6-fructosyltransferase, fructan exohydrolase, fructan 6-exohydrolase, cell wall invertase. Furthermore, aldo keto reductase and galactinol synthase were down-regulated. These observations suggest that NA wheat exposed to EC may allocate most of its carbon for respiration and growth, as indicated by the increase in biomass (Fig. S1), rather than allocation into storage carbon in the form of either starch or fructans. Although not statistically

significant, rates of respiration tended to be higher in NA Norstar grown at EC relative to ambient CO_2 (Table 1). Growth at EC may have improved chloroplast stability in CA wheat by the induction of a 29 kda putative RNA binding protein transcript. The expression of many chloroplast genes can be regulated by post-transcriptional RNA processing with ribonucleoproteins (Rochaix, 1995; Nakamura *et al.,* 2001; Ahsan *et al.,* 2010). The apparent insensitivity of the quantum requirement for PSII closure and the generation of NPQ in CA Norstar grown at EC (Fig. 3, Fig. 4) may be accounted for, in part, by the stabilization of chloroplast transcripts that are co-ordinately regulated (Fig. 7 ,Table S6). Several genes encoding ELIPS (7) and chaperonin proteins (9) were up-regulated by EC in CA wheat. Some members of the large family of ELIPs have non-light-harvesting functions but can also protect the thylakoids under stress conditions either by binding of free chlorophyll molecules and preventing the formation of free radicals and /or by acting as sinks for excitation energy (Adamska, 2004). Thus ELIPS have the ability to protect plants against damage of photoinhibition induced by high light stress (Adamska *et al.,* 1992; Pötter and Kloppstech, 1993). The up-regulation of several chaperonin transcripts (9) similar to Rubisco large subunit binding protein and classified in the protein folding category (Table 6 and Fig. 7) in CA wheat under EC may be very important since long term acclimation to high CO_2 can result in altered photosynthetic capacity in C3 crops associated with a decrease in the maximun Rubisco activity (Drake *et al.,* 1997; Ainsworth and Long, 2005). Thus, CA plants may be better adjusted to growth at EC as revealed by the up-regulation of transcripts involved in the protection and stability of chloroplasts.

Plant acclimation to EC usually results in a decrease in stomatal density (Woodward *et al.,* 2002; Hetherington and Woodward, 2003). The stomatal frequency of NA Norstar decreased by 30-40% when grown at elevated relative to ambient CO_2, which resulted in a decrease in stomatal conductance and transpiration

rates (Table 1). Consequently, WUE doubled in NA Norstar grown at elevated relative to ambient CO_2. In contrast, the stomatal frequency, stomatal conductance and transpiration rates of CA Norstar were rather insensitive to CO_2 concentrations (Table 1). This is due to the fact that CA at ambient CO_2 had already substantially reduced these parameters and enhanced WUE. Consequently, growth at EC had minimal impact on stomatal frequency, stomatal conductance and transpiration rates in CA Norstar (Table 1). It has been suggested that reduced stomatal density induced by changes in light irradiance is controlled by phytochrome interacting factors (PIFs) which consist of several closely related bHLH transcriptions factors (Monte *et al.,* 2007; Casson *et al.,* 2009). Down regulation of bHLH transcript by EC in NA (-7.9 fold; Table S3) and CA (-4.1; Table S6) wheat similar to the iron regulated transcription factor (iRO2) in graminaceous plants (Ogo *et al.,* 2006) was associated to the reduced stomatal density induced by EC. Although high CO_2 decreases stomatal conductance in NA wheat, the up-regulation (8 and 4 fold) of 2 transcripts encoding aquaporin proteins (Table S3) suggests a possible facilitation of small molecules diffusion in stomata. Aquaporins are large family of channel proteins present in the plasma and intracellular membranes of plants cells, where they facilitate the transport of water and other small solutes (urea, boric, acid, salicylic acid) and gases (ammonia, carbon dioxide) (Maurel *et al.,* 2008). The cDNA sequence of the most up-regulated (8 fold) aquaporin was annotated as a tonoplast intrinsic protein. However, analysis of the target signal peptide indicates that this aquaporin is a plasma membrane intrinsic protein (PIP). This aquaporin may participate in the facilitation of water or CO_2 diffusion and uptake during stress.

Several reports have shown that a previous exposure to one type of abiotic stress can lead to improved tolerance to another subsequent abiotic and biotic stress events (Rao *et al.,* 1995; Polle *et al.,* 1997; Pritchard *et al.,* 2000; Schwanz and Polle, 2001; Di Toppi *et al.,* 2002; Gillespie *et al.,* 2011). For example, cold acclimation of

overwintering grasses and cereals results in enhanced systemic resistance to infection by psychrophilic fungal pathogens called snow molds (Hiilovaara-Teijo *et al.,* 1999; Griffith and Yaish 2004). Similarly, exposure to EC affects herbivory-induced defense responses (Bidart-Bouzat *et al.,* 2005) and may decrease resistance to *Beetle japonica* (Casteel *et al.,* 2008) whereas increases in defence molecules such as tannins and gossypol in cotton have also been reported in response to EC (Wu *et al.,* 2011). Recently, De Lucia *et al.,* (2012) reported that alterations in plant phenology due to the combination of changes in temperature and [CO_2] affect interactions between plant and insect pollinators as well as between plant and insect herbivores. Our molecular analyses indicate that CA winter wheat grown at EC may be more tolerant to both biotic and abiotic stress than NA Norstar. Thus, the selection of winter wheat cultivars with increased innate tolerance to pathogens may be an important goal to improve plant productivity and avoid pathogen-related losses under EC conditions associated with future climate change.

4.6 MATERIALS AND METHODS

4.6.1 Plant materials and growth conditions

Seeds of winter (cv Norstar) wheat (*Triticum aestivum* L.) were grown in controlled environmental growth chambers (Model: GCW15 chamber, Environmental Growth Chambers, Chagrin Falls, OH, USA) at either ambient CO_2 (380 ± 10 μmol C mol^{-1}) or at EC (700 ± 25 μmol C mol^{-1}). NA plants were grown from seed at the day/night temperature regimes of 20/16°C and those of CA at 5/5°C from seed sowing. The plants were grown at a PPFD of 250 ± 20-μmol photons m^{-2} s^{-1}, 50% to 60% relative humidity and a 16/8 hr photoperiod. The CO_2 concentrations in the growth chamber were monitored continuously with a computer-controlled CO_2 infra-red gas analyzer (Model: WMA-4 CO_2 Analyzer, PP Systems International, Inc. Amesbury, MA, USA) installed in each CO_2 growth chamber. In addition, the temperature, relative humidity, irradiance level and photoperiod in each chamber were computer-controlled and monitored continuously. The seedlings were grown in coarse vermiculite in plastic pots at a density of three plants per pot and fertilized with Hoagland solution. In order to minimize rooting volume constraints and sink limitations, the plants were grown in 4L-sized plastic pots regardless of CO_2 conditions and growth temperatures.

Comparative growth kinetics were performed to assess the effects of EC on growth characteristics and to ensure that NA and CA plants were assessed at a comparable physiological stage of development for photosynthetic measurements as well as biochemical analyses. Regardless of CO_2 concentration, NA plants were harvested every week and CA plants every two weeks until the flag leaves were fully developed. Total tiller number, leaf number and leaf blade area were recorded on each harvest. Total root and shoot fresh biomass were weighed, and the tissues were dried at 80°C to constant weight for the determination of dry weight. Specific leaf

weight (SLW) was calculated as leaf dry weight in g m^{-2} leaf blade area. Leaf blade area was measured by using a LI-COR portable area meter (LI–3000A, LI-COR Biosciences, Lincoln, NE, USA). Chlorophyll a and b were quantified according to Arnon (1949).

4.6.2 CO2 gas exchange and measurements

CO_2 gas exchange rates were measured on fully expanded third leaves of 25-d-old NA and 75-d-old CA plants irrespective of growth CO_2. At these ages, the NA and CA plants were at comparable physiological stages of development based on growth kinetics (Dahal *et al.,* 2012b). All measurements of CO_2 gas exchange rates were carried out on fully expanded third leaves at either 380 or 700 µmol C mol^{-1} and at a measuring temperature of 20°C regardless of growth temperature. CO_2 gas exchange rates were measured by using the LI-COR portable infrared CO_2 gas analyzer (LI-6400 XRT Portable Photosynthesis System, LI-COR Biosciences, Lincoln, NE, USA). The apparent maximum quantum efficiency (Q) and the maximal photosynthetic capacity (A_{sat}) were determined as the maximum initial slope and the maximum light-saturated rates respectively from the light response curves by supplying 12 irradiance values over the range of 0 to 1500 µmol photons m^{-2} s^{-1} PPFD: Light response curves were measured from high to low light intensity with 8 min of waiting time between each measurement. CO_2 response curves were measured by providing 11 different CO_2 values over the range of 50 to 1200 µmol C mol^{-1} at a saturating irradiance of 1300 µmol photons m^{-2} s^{-1} PPFD. Rubisco-limited carboxylation efficiency (CE) was calculated as the maximum initial slope and the CO_2-saturated photosynthesis as the plateau of the curves. In addition, stomatal conductance and leaf transpiration rates were measured simultaneously with CO_2 gas exchange measurements at either 380 or 700 µmol C mol^{-1} and at 20°C regardless of growth temperature. Leaf water use efficiency was calculated as µmol CO_2 fixed per mol of stomatal conductance (A/gs). Stomatal density was estimated on both adaxial

and abaxial leaf surface using a stage micrometer ($1mm^2$) on a microscope (Leica ATC^{TM} 2000, Buffalo, NY, USA). Stomata were counted at a magnification of 100X.

4.6.3 Room Temperature Chl a Fluorescence

In vivo Chl a fluorescence was measured as previously described (Dahal *et al.*, 2012a; 2012b) to assess the effects of EC and cold acclimation on maximum potential rates of photosynthetic electron transport (ETR), excitation pressure (EP), which estimates the relative reduction state of PSII reaction centers and non-photochemical quenching (NPQ), an estimate of the capacity to dissipate excess energy as heat (Baker 2008). Chl a fluorescence was measured simultaneously with CO_2 gas exchange on fully expanded third leaves using LI-COR portable photosynthesis system (LI-6400 XRT, LI-COR Biosciences, Lincoln, NE, USA). All measurements of Chl a fluorescence were carried out by using the standard fluorescence leaf chamber (2 cm^2). The leaves were dark-adapted for 20 min prior to fluorescence measurements. Minimum fluorescence (F_o) was measured by illuminating dark adapted leaves with a low irradiance measuring beam (PPFD < 1 μmol photons m^{-2} s^{-1}) from a light emitting diode. Maximal fluorescence (F_m) was determined by applying second beam of saturating flash of light (PPFD >5000 μmol photons m^{-2} s^{-1}) for pulse duration of 0.8 s. Afterwards, an actinic light (PPFD 1300 μmol photons m^{-2} s^{-1}) was applied. Superimposed on the actinic beam was another saturating light flash (PPFD > 5000 μmol photons m^{-2} s^{-1}; 0.8 s) applied repetitively at 20 s intervals to determine maximal fluorescence in the light-adapted leaf (F_m'). Light adapted steady state fluorescence (F_s) was determined by measuring the level of fluorescence under steady state photosynthesis. Finally, minimal fluorescence (F_o') in the light-adapted leaf was measured by turning off the actinic light.

4.6.4 Microarray analysis

Twelve replicate pots with 3 plants per pot were grown at either NA or CA conditions at either ambient or EC. To minimize any possible chamber effects, the 12 replicate pots at each growth condition were distributed between two growth chambers. From the 6 pots in each growth chamber, 3 pots were randomly selected from which the fully expanded 3rd leaves from each plant were harvested, mixed and then divided into 3 replicate samples for microarray analyses.

Samples of Norstar winter wheat leaves were ground in dry ice to fine powder and total RNA was extracted with trizol (Invitrogen, Burlington, ON, CA). Total RNA was cleaned using RNeasy plant mini kit (Qiagen) and integrity was determined on agarose gel and on a bioanalyser (Agilent 2100). Synthesized cDNAs were transcribed to cRNAs with the 3'IVT labelling kit (Santa Clara, CA, USA) and hybridized to the Affymetrix wheat genome array (Santa Clara, Ca, USA) at the McGill University and Génome Québec Innovation Centre (Montreal, Qc, CA). The experimental design consisted of triplicate biological samples for each of the four growth conditions, 1: Non-acclimated (NA) at ambient CO_2 (AC) (NAAC); 2: Cold-acclimated (CA) at ambient CO_2 (AC) (CAAC); 3: Non-acclimated (NA) at elevated CO_2 (EC) (NAEC); 4: Cold-acclimated (CA) at elevated CO_2 (EC) (CAEC). Thus, a total of 3 samples from each of the 4 growth conditions described above were used for hybridizations.

Data analysis

Cel files obtained from chip images were imported in Flexarray (http://genomequebec.mcgill.ca/Flexarray); the data were normalized and corrected for background in R2.5.1 environment with the robust multi-array (RMA) method. To identify the probe-sets differentially regulated between two conditions, analysis of

variance (ANOVA) was performed on the expression of three replicates for each condition. We compared the transcriptome between 3 pairs of conditions to assess the effect of: 1) EC in non-acclimated plants (NAEC – NAAC); 2) EC in cold-acclimated plants (CAEC – CAAC) and 3) Cold acclimation (CAAC – NAAC). The probe-sets showing a fold change of 2 or more with a P value less or equal to 0.05 were selected for gene annotation with HarvEST: Affymetrix wheat 1 array 1.12 (harvest.ucr.edu). Categorical classification of differentially regulated probe-sets was performed with MAPMAN (http://gabi.rzpd.de/projects/MapMan) and through blast search and manual annotation retrieval at NCBI. Affymetrix indicates that the wheat array contains a collection of 61,127 probe-sets designed from 55,052 different transcripts. However, to avoid confusing terms, each annotated probe-set was considered as an independent gene since different regions of the same transcript are used to design different probe-sets.

4.6.5 Real-time PCR

To validate the micro-array data, real-time PCR was used. For cDNA synthesis, 5 µg of total RNA was digested with DNase I (Invitrogen Burlington, ON, CA) in a total volume of 50 µl. A volume of 10 µl of the DNase treated RNA was used for First-Strand cDNA synthesis with random hexamers and Superscript™ II Reverse Transcriptase as described (Invitrogen Burlington, ON, CA). The cDNAs obtained were diluted ten-fold in deionized water. Quantitative real-time PCR was performed on a lightcycler 480 (Roche). Briefly the reaction mixture comprises 2 µl of the diluted cDNAs, 12,5 µl of SYBR-Green PCR Mastermix (SABiosciences, MD 21703 USA) 1,5 µl of primers at 10 µM (50:50 mix of forward and reverse primers at 10 µM each) and 6,5 µl of H_2O. For qRT-PCR validation, the target sequence obtained from Affymetrix were BLASTED in HarvEST or NCBI and the

corresponding cDNAs were used to design specific primers with Beacon design software (PREMIER Biosoft International Palo alto, CA, USA) or ready to use primers from chloroplast database for higher plants (Heinze, 2007). The primers used for gene amplification are listed in Table S1. The Amplification efficiency was determined with a dilution series (100, 50, 25, 12.5, 6.25, 3.12 and 1.56 ng) of the cDNA pool from all the samples. The mix of primers, SYBR Green and water were added to the plate containing the template cDNA. The reactions were pre-incubated for 5min, at 95 ^{0}C. The amplification step was set at 95 ^{0}C for 10s with a target temperature of 3 ^{0}C below the calculated primer Tm and annealing was set at 72 ^{0}C for 5s. For melting curves, the temperature was set at 95 ^{0}C for 5s, 65 ^{0}C for 1 min and 97 ^{0}C continuous. The reactions were cooled at 40 ^{0}C for 10s. Melting curves of amplified samples were analysed and no primer dimers were detected. Two biological samples of each condition with 3 technical replicates were analyzed by qRT-PCR. The 18S ribosomal RNA was used as an internal standard for normalization of expression levels with the delta-delta-Ct method for the calculation of relative expression (Livak and Schmittgen, 2001).

Table 4.1: Effects of cold acclimation and elevated CO2 on growth, physiological and stomatal characteristics of Norstar winter wheat. Plants were grown at either ambient CO2 (380 µmol C mol-1) or elevated CO2 (700 µmol C mol-1) and at either 20/16°C or 5/5°C. Data represent the mean of nine plants from three different pots ± SD. Significant differences of the means are indicated by the superscripted letters ($P \leq 0.05$).

Growth, physiological and stomatal characteristics	Growth temperature (°C)/growth CO_2 (µmol C mol^{-1})			
	20/380	20/700	5/380	5/700
Exponential growth rates	$0.222 \pm .001^{c}$	0.229 ± 0.002^{d}	0.072 ± 0.002^{a}	0.076 ± 0.001^{b}
Total Shoot Biomass (mg DW $plant^{-1}$)	257 ± 20^{ab}	328 ± 16^{c}	226 ± 27^{a}	301 ± 20^{bc}
Specific leaf weight (gDW m^{-2} leaf area)	30 ± 5^{a}	34 ± 6^{a}	72 ± 9^{b}	77 ± 6^{b}
Chlorophyll (mg m^{-2} leaf area)	581 ± 31^{a}	698 ± 44^{b}	761 ± 65^{b}	736 ± 30^{b}
Leaf protein (g m^{-2} leaf area)	3.36 ± 0.24^{a}	2.98 ± 0.17^{a}	8.33 ± 0.98^{b}	7.55 ± 0.60^{b}
R_{dark} (µmol C evolved $m^{-2}s^{-1}$)	3.07 ± 0.23^{a}	3.41 ± 0.31^{ab}	3.88 ± 0.18^{b}	3.97 ± 0.43^{b}
Adaxial stomatal density (stomata mm^{-2})	109 ± 3^{b}	73 ± 4^{a}	71 ± 12^{a}	78 ± 12^{a}
Abaxial stomatal density (stomata mm^{-2})	122 ± 18^{b}	67 ± 6^{a}	76 ± 11^{a}	68 ± 16^{a}
Stomatal conductance (mol $m^{-2}s^{-1}$)	0.59 ± 0.06^{c}	0.39 ± 0.03^{b}	0.35 ± 0.02^{ab}	0.31 ± 0.02^{a}
Transpiration (mmol H_2O m^{-2} s^{-1})	3.28 ± 0.19^{c}	2.38 ± 0.12^{b}	2.05 ± 0.15^{a}	2.23 ± 0.25^{ab}
Water Use Efficiency (A/g_s)	28 ± 4^{a}	64 ± 6^{b}	48 ± 9^{b}	89 ± 21^{c}

Table 4.2: Categorical distribution of known genes regulated by elevated CO2 in non-acclimated plants.

Category	Number of genes
Stress,biotic	42
RNA, regulation of transcription	40
Carbohydrate metabolism	38
Transport	25
Stress abiotic	24
Oxidation-reduction process	23
Secondary metabolism	19
Hormone metabolism	17
Protein postranslational modification	15
Amino acid metabolism	11
Lipid metabolism	11
Protease inhibitor	11
Signaling	11
DNA repair, DNA synthesis/chromatin structure	9
Photosynthesis	9
Protein degradation	9
Development	6
Cell organisation	5
RNA binding	5
Metal handling binding, chelation and storage	4
N-metabolism, nitrate metabolism, NR	3
Cell wall	2
Mitochondrial electron transport	2
Nucleotide metabolism, degradation	2
RNA processing ribonucleases	2
TCA cycle	2

Table 4.3: MAPMAN functional categories of genes co-ordinately regulated by elevated carbon dioxide in non-acclimated wheat and having quite similar fold change within a category. The genes retained are for those functional groups that exhibited a significantly (Benjamini Hochberg corrected wilcoxon rank sum test; $p \leq 0.05$) different behaviour in terms of expression profiles compared with all the remaining groups during exposure to elevated CO_2.

Probe-sets	Functional category and gene description	Fold change	UniProt Accn
	Stress biotic		
Ta.9226.1.S1_at	Wheatwin-2	-5.50	O64393
Ta.221.1.S1_at	Chitinase II	-5.19	Q9XEN3
Ta.26983.1.A1_at	Chitinase 8	-4.97	Q7XCK6
TaAffx.106304.1.S1_at	Thaumatin-like protein TLP4	-4.80	Q946Z1
Ta.97.2.S1_x_at	Protein WIR1A	-4.63	Q01482
Ta.27762.1.S1_x_at	Pathogenesis-related protein 1A/1B	-4.51	P32937
Ta.2278.2.S1_x_at	Chitinase IV	-3.87	Q9XEN6
Ta.2278.2.S1_a_at	Chitinase IV	-3.84	Q9XEN6
TaAffx.108556.1.S1_at	Putative uncharacterized protein	-3.73	B4FS23
Ta.24501.1.S1_at	Pathogenesis-related protein 1A/1B	-3.65	P32937
Ta.21556.1.S1_x_at	Protein WIR1B	-3.53	Q01481
Ta.13.1.S1_at	WIR1 protein	-3.41	Q41581
Ta.21556.1.S1_at	Protein WIR1B	-3.30	Q01481
Ta.2278.1.S1_x_at	Chitinase IV	-3.28	Q9XEN6
Ta.2278.2.S1_at	Chitinase IV	-2.96	Q9XEN6
Ta.29307.1.A1_s_at	Os10g0503300 protein	-2.91	Q0IWK4
Ta.2278.3.S1_x_at	Chitinase II	-2.89	Q9XEN3
TaAffx.108556.1.S1_x_at	Putative uncharacterized protein	-2.83	B4FS23
Ta.13785.1.S1_at	Xylanase inhibitor	-2.74	A7BJ77
Ta.224.1.S1_at	Chitinase IV	-2.72	Q9XEN6
Ta.999.1.S1_at	Taxadien-5-alpha-ol O-acetyltransferase	-2.62	B6TZX2
Ta.30501.1.S1_at	Chitinase	-2.60	C8CG67
Ta.23322.1.S1_s_at	Thaumatin-like protein TLP8	-2.59	Q946Y8
Ta.23322.2.S1_at	Thaumatin-like protein TLP8	-2.56	Q946Y8
Ta.192.1.S1_at	Putative uncharacterized protein	-2.50	Q41523
Ta.97.1.S1_at	Protein WIR1B	-2.34	Q01481
Ta.231.1.S1_x_at	PR17c	-2.25	A7YA60
TaAffx.28302.2.S1_at	Dirigent-like protein	-2.18	Q53NP6
Ta.4383.1.A1_at	Putative uncharacterized protein	-2.02	C4IYK7
Ta.25549.1.S1_at	NBS-LRR disease resistance protein, putative	-2.78	Q2R8J2
Ta.22687.1.A1_at	Putative uncharacterized protein	-2.78	C5X502
	Carbohydrate metabolism		
Ta.4494.1.S1_x_at	Beta-amylase	-3.57	Q10RZ1
Ta.4494.1.S1_at	Beta-amylase	-2.50	Q10RZ1
TaAffx.42783.1.A1_s_at	Beta-amylase	-2.16	B6SVZ0
Ta.10107.1.S1_at	Fructokinase-1	-2.05	Q0JGZ6
Ta.9641.1.A1_a_at	Fructan 6-exohydrolase	-4.37	Q2UXF7

Ta.2789.1.S1_a_at	Sucrose:fructan 6-fructosyltransferase	-4.20	Q96466
Ta.2789.2.S1_at	Sucrose:fructan 6-fructosyltransferase	-3.54	Q96466
Ta.2789.2.S1_x_at	Sucrose:fructan 6-fructosyltransferase	-3.35	Q96466
Ta.7151.1.S1_x_at	Fructan exohydrolase	-2.87	Q4W8R0
Ta.9765.1.S1_at	Cell wall invertase	-2.53	Q2QI10
Ta.2789.1.S1_at	Sucrose:fructan 6-fructosyltransferase	-5.28	Q96466
Ta.3475.1.A1_at	Fructan:fructan 1-fructosyltransferase	-3.09	B5TK36
Ta.11152.2.A1_a_at	Sucrose synthase (Fragment)	-3.89	Q8LPU9
Ta.2491.1.S1_at	ADP, ATP carrier protein 2	-2.05	Q2R030
	RNA regulation of transcription		
Ta.12422.1.S1_at	Two-component response regulator-like PRR1	2.06	Q689G9
Ta.29499.1.A1_at	Two-component response regulator-like PRR1	2.24	Q689G9
Ta.12422.2.S1_a_at	Two-component response regulator-like PRR1	2.26	Q689G9
Ta.13464.1.S1_s_at	Two-component response regulator-like PRR1	2.37	Q689G9
TaAffx.86800.1.S1_at	Two-component response regulator-like PRR1	3.43	Q689G9
Ta.12422.3.S1_a_at	Two-component response regulator-like PRR1	3.51	Q689G9
TaAffx.86800.1.S1_x_at	Two-component response regulator-like PRR1	3.99	Q689G9
	RNA binding		
Ta.260.1.S1_at	RNA-binding protein	2.12	Q9AXN2
TaAffx.33515.2.A1_x_at	Glycine-rich RNA binding protein	2.14	Q6ASX7
Ta.28306.1.S1_at	Putative glycine-rich RNA-binding protein 2	2.17	B5KLZ7
TaAffx.13219.1.S1_at	Os03g0836200 protein	2.65	Q75LJ7
TaAffx.33515.1.A1_at	Putative glycine-rich RNA-binding protein 2	2.88	B5KLZ7
TaAffx.13219.2.S1_s_at	Os03g0836200 protein	3.39	Q75LJ7
	Hormone metabolism		
TaAffx.128684.1.S1_at	12-oxophytodienoic acid reductase 1	2.23	A3QQM8
Ta.1207.1.S1_s_at	12-oxophytodienoic acid reductase 1	2.78	A3QQM8
TaAffx.128684.1.S1_x_at	12-oxophytodienoic acid reductase 1	3.07	A3QQM8
Ta.1207.1.S1_x_at	12-oxophytodienoic acid reductase 1	3.95	A3QQM8
Ta.1207.1.S1_at	12-oxophytodienoic acid reductase 1	3.95	A3QQM8
	Abiotic stress		
Ta.20804.2.S1_x_at	Germin-like protein 1	4.99	O49871
Ta.28351.1.S1_x_at	Germin-like protein 1	6.64	O49871
Ta.28351.1.S1_s_at	Germin-like protein 1	6.70	O49871
Ta.28351.1.S1_at	Germin-like protein 1	8.12	O49871

Table 4.4: Defense-related genes down-regulated by elevated CO2 in non-acclimated wheat.

Probe-sets	Gene description	Fold change	UniProt Accn
Ta.21348.1.S1_s_at	sulfur-rich/thionin-like protein	-90.29	Q41521
Ta.21348.1.S1_x_at	sulfur-rich/thionin-like protein	-58.06	Q41521
Ta.21348.2.S1_at	sulfur-rich/thionin-like protein	-51.11	Q41521
ta.9107.1.s1_x_at	1-aminocyclopropane-1-carboxylate oxidase	-2.47	Q9AYS8
ta.9107.2.s1_a_at	1-aminocyclopropane-1-carboxylate oxidase	-2.72	Q9AYS8
Ta.28.1.S1_at	1,3-beta glucanase	-2.40	Q84LJ5
ta.24336.1.s1_at	4-hydroxyphenylpyruvate dioxygenase	-3.32	HPPD
ta.24336.1.s1_x_at	4-hydroxyphenylpyruvate dioxygenase	-3.14	HPPD
taaffx.143995.11.s1_s_at	APETALA2-like protein	-2.33	B0FTT5
Ta.25053.1.S1_at	Basic pathogenesis-related protein PR5	-2.81	O23997
Ta.21354.1.A1_x_at	Beta-1,3-glucanase	-2.79	Q9XEN5
Ta.22427.1.A1_x_at	Beta-1,3-glucanase	-2.18	Q9XEN5
Ta.225.1.S1_at	Beta-1,3-glucanase	-2.40	Q9XEN7
Ta.21354.1.A1_at	Beta-1,3-glucanase	-2.30	Q9XEN5
Ta.223.1.S1_at	Beta-1,3-glucanase	-2.91	Q9XEN5
ta.8484.1.a1_at	Beta-carotene hydroxylase	-2.16	Q1XBU3
Ta.21297.1.S1_at	Beta-glucanase	-2.42	Q7M1K2
Ta.4601.2.S1_at	Beta-glucosidase	-4.11	Q1XH04
TaAffx.12607.1.S1_at	Beta-glucosidase 16	-4.98	BGL16
Ta.23208.1.S1_at	Beta-glucosidase 5	-2.29	BGL05
ta.2585.1.s1_at	Betaine aldehyde dehydrogenase (Fragment)	-2.52	Q5KSN8
TaAffx.26815.1.S1_at	BLN1-2	-2.48	B8X453
TaAffx.124475.1.A1_at	BLT14.2 protein	-2.44	Q40033
Ta.13371.1.S1_at	Bowman-Birk type trypsin inhibitor	-9.78	IBB
Ta.13371.1.S1_x_at	Bowman-Birk type trypsin inhibitor	-10.93	IBB
Ta.13371.2.S1_x_at	Bowman-Birk type trypsin inhibitor	-3.20	IBB
ta.336.2.s1_at	Caffeic acid O-methyltransferase	-3.11	Q84N28
Ta.336.2.S1_at	Caffeic acid O-methyltransferase	-3.11	Q84N28
Ta.336.1.S1_x_at	Caffeic acid O-methyltransferase	-2.69	Q84N28
ta.10909.1.s1_s_at	Carboxyl-terminal-processing protease	-4.12	B6T6L2
ta.10909.1.s1_x_at	Carboxyl-terminal-processing protease	-6.66	B6T6L2
Ta.25518.1.S1_at	Cf2/Cf5 disease resistance protein homolog	-2.80	Q9SWE6
ta.11832.1.s1_at	Chaperone protein dnaJ	-2.18	B6THE8
Ta.30501.1.S1_at	Chitinase	-2.60	C8CG67
Ta.26983.1.A1_at	Chitinase 8	-4.97	CHI8
Ta.2278.3.S1_x_at	Chitinase II	-2.89	Q9XEN3
Ta.221.1.S1_at	Chitinase II	-5.19	Q9XEN3
Ta.224.1.S1_at	Chitinase IV	-2.72	Q9XEN6
Ta.2278.1.S1_x_at	Chitinase IV	-3.28	Q9XEN6
Ta.2278.2.S1_a_at	Chitinase IV	-3.84	Q9XEN6
Ta.2278.2.S1_at	Chitinase IV	-2.96	Q9XEN6
Ta.2278.2.S1_x_at	Chitinase IV	-3.87	Q9XEN6
ta.1183.1.s1_at	Cinnamoyl-CoA reductase	-3.85	A8DNN6
ta.6747.1.s1_at	Cinnamyl alcohol dehydrogenase	-2.25	Q8S411
taaffx.11059.1.a1_s_at	Cinnamyl alcohol dehydrogenase	-2.22	Q8S411
ta.22744.1.s1_at	Copper chaperone for superoxide dismutase	-4.88	B6TLT7
Ta.21342.1.S1_x_at	Endochitinase	-2.62	Q41539
ta.2834.1.s1_at	Glucan endo-1,3-beta-glucosidase	-2.00	B6SJQ8
taaffx.107485.1.s1_at	Glucomannan 4-beta-mannosyltransferase	-2.76	CSLA1

ta.28354.1.s1_at	Glutathione transferase	-2.52	Q8RW00
ta.28354.3.s1_x_at	Glutathione transferase	-2.10	Q8RW00
Ta.25611.1.S1_at	GRAB2 protein	-2.38	Q9ZRZ2
TaAffx.103486.1.A1_x_at	GRAB2 protein	-2.47	Q9ZRZ2
Ta.16038.1.S1_at	HVA22-like protein e, putative, expressed	-2.60	Q2R3W9
ta.5251.1.a1_at	Isoform MYBAS1-1 of Myb-related protein MYBAS1	-2.02	Q53NK6
Ta.18186.1.A1_at	Lipid transfer protein	-2.56	Q6S509
taaffx.90316.1.s1_at	Lipoxygenase	-2.30	A3ALS8
ta.23763.1.s1_at	Lipoxygenase (Fragment)	-6.85	Q41520
ta.9600.1.s1_x_at	Low molecular mass early light-inducible protein	-3.44	ELI9
ta.26049.1.s1_a_at	Myb-related protein Hv1	-20.09	MYB1
Ta.25549.1.S1_at	NBS-LRR disease resistance protein, putative, expressed	-2.78	Q2R8J2
Ta.21646.1.S1_at	Non-specific lipid-transfer protein	-3.07	Q75GN2
Ta.21646.1.S1_x_at	Non-specific lipid-transfer protein	-3.76	Q75GN2
Ta.21957.1.S1_at	Non-specific lipid-transfer protein	-4.22	Q7XIG9
Ta.13070.1.S1_at	Non-specific lipid-transfer protein	-2.11	Q1KMU9
TaAffx.96188.1.S1_at	Non-specific lipid-transfer protein	-2.11	Q75GN2
TaAffx.96188.2.S1_at	Non-specific lipid-transfer protein	-2.36	Q75GN2
Ta.5812.1.A1_at	Papain-like cysteine proteinase	-2.18	B4ESF1
Ta.30739.2.S1_at	Pathogenesis-related 1a	-3.52	Q3S4I
Ta.22619.1.S1_at	Pathogenesis-related protein	-2.23	Q84QC7
Ta.22619.1.S1_x_at	Pathogenesis-related protein	-2.38	Q84QC7
Ta.24501.1.S1_at	Pathogenesis-related protein 1A/1B	-3.65	PR1A
Ta.27762.1.S1_x_at	Pathogenesis-related protein 1A/1B	-4.51	PR1A
Ta.278.1.S1_at	Pathogenesis-related protein PRB1-2	-2.70	PR12
Ta.278.1.S1_x_at	Pathogenesis-related protein PRB1-2	-2.93	PR12
Ta.5385.1.S1_at	Peroxidase 1	-7.79	PER1
Ta.9420.1.S1_at	Peroxidase 1	-2.50	PER1
Ta.20429.1.S1_at	Phenylalanine ammonia-lyase	-2.24	Q43664
Ta.28736.1.S1_at	Phenylalanine ammonia-lyase	-2.30	C5YCD7
ta.20776.1.s1_at	Phytoene synthase 7A	-2.73	C1IP77
Ta.231.1.S1_x_at	PR17c	-2.25	A7YA60
ta.13798.1.s1_at	Probable cinnamyl alcohol dehydrogenase	-2.04	CADH
taaffx.28494.1.s1_s_at	Probable indole-3-acetic acid-amido synthetase	-2.49	GH35
ta.27816.1.a1_at	Protein binding protein	-2.01	B6UCS6
ta.74.1.s1_at	Protein disulfide-isomerase	-2.10	PDI
ta.796.1.s1_x_at	Protein disulfide-isomerase	-2.04	PDI
Ta.3133.1.S1_x_at	Protein WIR1A	-4.22	WIR1A
Ta.5518.1.S1_at	Protein WIR1A	-2.44	WIR1A
Ta.97.2.S1_x_at	Protein WIR1A	-4.63	WIR1A
Ta.21556.1.S1_at	Protein WIR1B	-3.30	WIR1B
Ta.21556.1.S1_x_at	Protein WIR1B	-3.53	WIR1B
Ta.97.1.S1_at	Protein WIR1B	-2.34	WIR1B
taaffx.59343.1.s1_at	Putative acyltransferase	-2.58	Q8W3C6
Ta.21350.2.S1_at	Putative Bowman-Birk wound-induced protease inhibitor	-3.81	B7SKZ2
Ta.30711.1.S1_x_at	Putative Bowman-Birk wound-induced protease inhibitor	-2.88	B7SKZ2
ta.27228.1.s1_at	Putative galactoside 2-alpha-L-fucosyltransferase	-2.01	Q6Z6F4
taaffx.129720.2.s1_at	Putative pectine lyase RepID=	-2.84	Q6H8B0
ta.7524.1.a1_at	Putative TdLFC65 protein (Fragment)	-5.02	C4WYK0
ta.5331.1.a1_x_at	Serine/threonine protein kinase	-2.58	
taaffx.128570.1.s1_at	Serine/threonine protein kinase	-2.50	A3FPG3
ta.10555.1.a1_at	Serine/threonine protein kinase	-2.57	A3FPG3
ta.5331.1.a1_a_at	Serine/threonine protein kinase	-2.07	
taaffx.38062.1.a1_at	Sesquiterpene synthase	-2.54	Q6ZJL3
ta.30553.1.s1_at	SKIP interacting protein 20	-2.50	B8Q8A6
ta.3344.1.s1_at	Strictosidine synthase	-2.18	B6TK54
ta.166.1.s1_a_at	Superoxide dismutase [Cu-Zn]	-2.87	O24400
ta.8051.1.s1_at	Superoxide dismutase	-2.26	Q5FB28
taaffx.12557.1.a1_at	Terpene synthase 7	-6.03	B6SPA6
Ta.959.1.S1_at	Thaumatin-like protein	-2.33	Q41584
TaAffx.106304.1.S1_at	Thaumatin-like protein TLP4	-4.80	Q946Z1

Ta.191.1.S1_at	Thiol protease	-33.47	Q41522
TaAffx.28364.1.S1_at	Triticum aestivum sulfur-rich/thionin-like protein	-2.10	Q41521
Ta.21267.1.S1_s_at	Wali3 protein	-3.48	Q43663
Ta.5024.1.S1_x_at	Wali3 protein	-6.20	Q43663
Ta.21350.1.S1_x_at	Wali5 protein	-7.58	Q43665
taaffx.85861.1.s1_at	Wall-associated kinase-like	-3.52	Q6H7K3
Ta.11087.1.S1_at	WIR1 protein	-2.59	Q41581
Ta.13.1.S1_at	WIR1 protein	-3.41	Q41581
ta.8614.1.s1_at	WRKY45 transcription factor	-3.08	A3RG93

Table 4.5: Categorical distribution of the known genes regulated by elevated CO2 in cold-acclimated plants.

Category	Number of genes
Stress abiotic	19
Oxidation-reduction process	16
RNA, regulation of transcription	14
Protein degradation	12
Signaling	11
Protein folding	9
Secondary metabolism	9
Carbohydrate metabolism	9
Transport	7
Photosynthesis	7
Development	6
Protein postranslational modification	6
Protein synthesis	5
RNA processing	5
RNA binding	5
Aminoacid metabolism	5
Hormone metabolism	4
Stress-biotic	3
Protease inhibitor	2

Table 4.6: MAPMAN functional categories of genes co-ordinately regulated by elevated carbon dioxide in cold-acclimated wheat and having quite similar fold change within a category. The genes retained are for those functional groups that exhibited a significantly (Benjamini Hochberg corrected wilcoxon rank sum test; $p \leq 0.05$) different behaviour in terms of expression profiles compared with all the remaining groups during exposure to elevated CO2.

Probe-sets	Functional category and gene description	Fold change	UniProt Accn
	Signaling		
Ta.18362.1.A1_at	High molecular mass early light-inducible protein HV58	6.49	P14895
Ta.23419.1.S1_x_at	High molecular mass early light-inducible protein HV58	4.95	P14895
Ta.25398.1.S1_at	Low molecular mass early light-inducible protein HV90	3.04	P14897
Ta.26973.1.S1_at	Low molecular mass early light-inducible protein HV90	6.96	P14897
Ta.26997.1.S1_at	High molecular mass early light-inducible protein HV58	7.05	P14895
Ta.9600.1.S1_x_at	Low molecular mass early light-inducible protein HV90	4.44	P14897
TaAffx.128418.14.A1_at	High molecular mass early light-inducible protein HV58	6.01	P14895
	Secondary Metabolism		
Ta.14243.1.A1_at	Flavanone 3-hydroxylase (Fragment)T	8.18	B5L5S6
Ta.28700.1.S1_at	Chalcone synthase	5.06	Q2L6K4
Ta.9172.1.S1_at	Chalcone synthase 1	4.38	Q9XGX2
Ta.9172.1.S1_x_at	Chalcone synthase 1	4.47	Q9XGX2
Ta.9983.1.S1_s_at	Dihydroflavonol 4-reductase	4.85	Q6UB70
Ta.9983.2.S1_at	Dihydroflavonol 4-reductase	9.01	Q6UB70
	Protein folding		
Ta.1321.1.S1_at	Chaperonin	3.27	B6T8U7
Ta.1321.1.S1_s_at	Chaperonin	2.69	B6T8U7
Ta.13250.1.S1_a_at	Chaperonin	2.42	B4F848
Ta.13250.2.S1_x_at	Chaperonin	2.68	B4F848
Ta.28883.1.A1_at	Chaperonin	2.53	B6T8U7
Ta.6964.1.S1_at	Chaperonin CPN60-1	2.04	P29185
Ta.7981.1.S1_s_at	Sb04g035040	2.12	C5XTN5
Ta.9305.1.S1_at	Putative chaperonin 60 beta (Os06g0114000 protein)	2.49	Q9LWT6

Table 4.7: Defense-related genes down-regulated by elevated CO2 in non-acclimated (NAEC-NAAC) wheat but up-regulated by cold-acclimation at ambient CO2 (CAAC-NAAC).

Probe-sets	Gene description	NAEC-NAAC	CAAC-NAAC	UniProt Accn
		Fold change	Fold change	
TaAffx.107485.1.S1_at	Glucomannan 4-beta-mannosyltransferase	-2.76	20.75	Q7PC76
Ta.27347.1.S1_at	Os07g0637300 protein	-2.62	2.59	Q8H5R7
Ta.28736.1.S1_at	Phenylalanine ammonia-lyase	-2.30	3.11	C5YCD7
Ta.8390.1.S1_at	Putative uncharacterized protein Sb05g022350	-3.17	25.02	C5Y4X4
Ta.21297.1.S1_at	Beta-glucanase	-2.42	27.64	Q7M1K2
Ta.223.1.S1_at	Beta-1,3-glucanase	-2.91	27.37	Q9XEN5
Ta.22427.1.A1_x_at	Beta-1,3-glucanase	-2.18	34.38	Q9XEN5
Ta.225.1.S1_at	Beta-1,3-glucanase	-2.40	34.59	Q9XEN7
Ta.28354.3.S1_x_at	Glutathione transferase	-2.10	20.47	Q8RW00
Ta.28546.1.A1_at	Putative uncharacterized protein	-2.07	2.78	C4J0S0
Ta.5460.1.S1_s_at	Os11g0107600	-3.04	2.13	Q0IV74
Ta.11206.1.A1_at	Putative uncharacterized protein	-2.69	4.04	C5XLP1
Ta.3942.1.S1_a_at	Os05g0585500 protein	-2.13	3.30	Q6I587
TaAffx.59343.1.S1_at	Putative acyltransferase	-2.58	2.11	Q8W3C6
Ta.21555.1.S1_at	Os07g0587500 protein	-2.01	2.35	Q6ZIQ0
Ta.28354.1.S1_at	Glutathione transferase	-2.52	11.13	Q8RW00
Ta.8614.1.S1_at	WRKY45 transcription factor	-3.08	6.55	A3RG93
Ta.5331.1.A1_a_at	Serine/threonine protein kinase	-2.07	32.16	A3FPG3
Ta.8484.1.A1_at	Beta-carotene hydroxylase	-2.16	3.53	Q1XBU3
Ta.6247.2.S1_at	Os09g0252100 protein (Fragment)	-2.06	2.39	Q0J366
Ta.24934.3.S1_at	Putative uncharacterized protein Sb01g031160	-2.09	71.77	C5WUL5
Ta.5623.2.S1_a_at	Putative uncharacterized protein	-2.58	5.87	A2X1J7
Ta.13798.1.S1_at	Probable cinnamyl alcohol dehydrogenase	-2.04	8.26	O22380
Ta.336.2.S1_at	Caffeic acid O-methyltransferase	-3.11	2.12	Q84N28
Ta.3507.2.S1_a_at	Putative uncharacterized protein Sb08g020950	-2.09	2.51	C5YRJ2

Ta.20429.1.S1_at	Phenylalanine ammonia-lyase (Fragment)	-2.24	12.00	Q43664
Ta.336.1.S1_x_at	Caffeic acid O-methyltransferase	-2.69	2.80	Q84N28
Ta.3976.1.S1_at	Os04g0581000 protein	-4.45	21.31	Q7FAL4
Ta.5623.1.S1_x_at	Putative uncharacterized protein	-2.21	3.66	A2X1J7
Ta.27816.1.A1_at	Protein binding protein	-2.01	4.17	B6UCS6
Ta.9600.1.S1_x_at	Low molecular mass early light-inducible protein	-3.44	9.02	P14897
Ta.10555.1.A1_at	Serine/threonine protein kinase	-2.57	11.74	A3FPG3
Ta.27228.1.S1_at	Os02g0763900 protein	-2.01	3.82	Q6Z6F4
Ta.5331.1.A1_x_at	Serine/threonine protein kinase	-2.58	21.76	A3FPG3
Ta.5385.1.S1_at	Peroxidase 1	-7.79	4.79	P27337
TaAffx.128570.1.S1_at	Serine/threonine protein kinase	-2.50	6.27	A3FPG3
TaAffx.26668.1.S1_at	Putative uncharacterized protein	-2.39	5.26	C5WPZ1
Ta.26901.1.S1_at	Os06g0557100 protein	-2.21	5.13	Q5Z7H5
Ta.12127.1.A1_at	Os02g0615800 protein	-3.08	2.45	Q6K7X0
Ta.7511.1.S1_at	Os03g0329700	-3.04	5.69	UPI0000E1205B
TaAffx.85861.1.S1_at	Wall-associated kinase-like	-3.52	4.20	Q6H7K3
TaAffx.83027.1.S1_at	Os05g0135100	-2.10	12.51	Q0DKY8
TaAffx.82108.1.S1_at	Os01g0115700	-2.17	8.01	UPI0000DD891C
TaAffx.82108.1.S1_x_at	Os01g0115700	-2.05	7.24	UPI0000DD891C
Ta.8773.1.S1_x_at	Os02g0710900 protein	-2.05	4.23	Q6ZFX8
Ta.8773.2.S1_x_at	Os02g0710900 protein	-2.11	4.58	
Ta.22687.1.A1_at	Putative uncharacterized protein	-2.78	39.82	C5X502
Ta.25549.1.S1_at	NBS-LRR disease resistance protein,	-2.78	4.57	Q2R8J2

Table 4.8. Quantitative real time PCR (qRT-PCR) validation of selected genes from the microarray analyses. Fold change (FC) of genes regulated by elevated CO2 in 1) non acclimated wheat (NAEC-NAAC); 2) cold acclimated wheat (CAEC-CAAC) and 3) regulated by cold acclimation at ambient CO2 (CAAC-NAAC). STD dcp: standard deviation of crossing point difference.

Probeset	Annotation	NAEC-NAAC				CAEC - CAAC				CAAC-NAAC			
		Microarray		qRT-PCR		Microarray		qRT-PCR		Microarray		qRT-PCR	
		FC	P-value	FC	STD dcp	FC	P-value	FC	STD dcp	FC	P-value	FC	STD dcp
Ta.439.1.S1_at	Fructose-1,6-bisphosphatase, chloroplastic	1.29	0.002	-1.43	0.22	1.18	0.006	1.00	0.23	1.21	0.009	-1.33	0.21
Ta.278.1.S1_at	Pathogenesis-related protein PRB1-2	-2.70	0.151	-3.33	0.34	1.14	0.001	2.49		16.08	0.007	52.00	0.32
Ta.24501.1.S1_at	Pathogenesis-related protein 1A/1B (Thaumatin domain)	-3.65	0.004	-10.00	0.24	-1.10	0.194	1.80	0.42	23.86	0.000	25.50	0.34
TaAffx.45277.1.S1_x_at	Phenylalanine ammonia-lyase	1.09	0.782	-1.56	0.48	-1.70	0.001	-1.12	0.46	36.87	0.000	20.40	0.47
Ta.28917.1.S1_x_at	Cold acclimation protein WCOR518	2.07	0.026	3.29	0.38	1.56	0.000	1.40	0.19	5.31	0.001	7.63	0.32
Ta.12663.1.S1_at	Ice recrystallization inhibition protein	2.04	0.139	1.92	0.39	1.44	0.001	3.10	0.23	27.76	0.001	16.10	0.35
Ta.3471.1.S1_at	Sucrose-phosphate synthase	1.03	0.826	1.00	0.34	1.12	0.252	-1.08	0.27	-1.22	0.059	-1.12	0.21
TaAffx.128757.1.S1_at	Photosystem Q(B) protein	1.16	0.246	3.89	0.26	-1.58	0.112	1.88	0.37	-1.15	0.553	-2.00	0.38
TaAffx.128414.37.S1_at	Ribulose bisphosphate carboxylase large chain	-1.03	0.758	2.24	0.44	1.02	0.895	-1.25	0.32	1.05	0.613	1.09	0.44
Ta.221.1.S1_at	Chitinase II	-5.19	0.001	-1.85	0.39	1.36	0.016	3.35	0.34	22.47	0.000	9.66	0.29

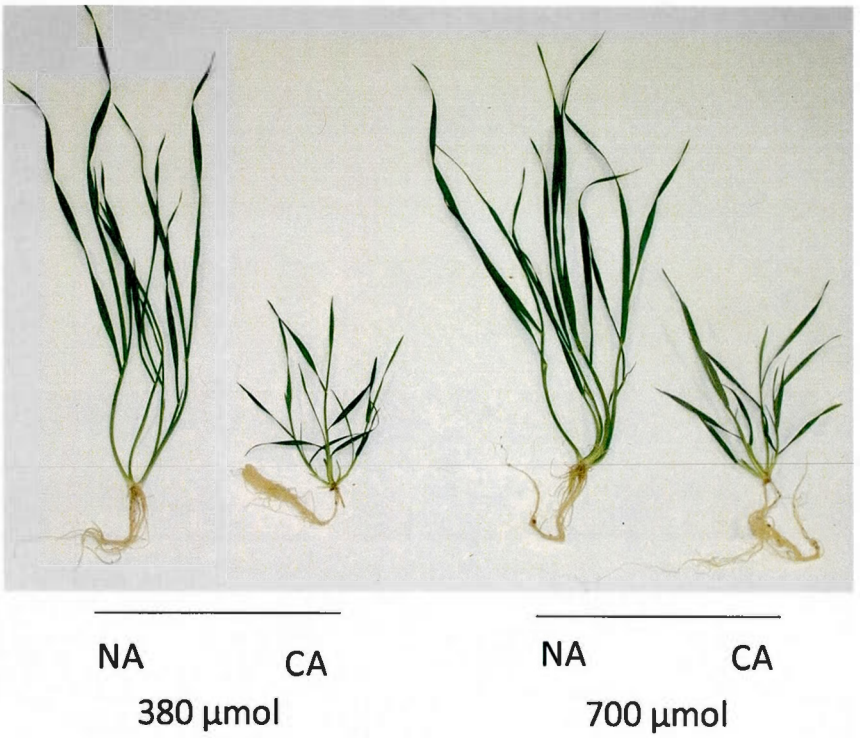

Figure 4.1: Effects of elevated CO2 and growth temperatures on plant morphology and growth habit of Norstar winter wheat. Photographs were taken from 25-d-old non-acclimated (NA) and 75-d-old cold acclimated (CA) Norstar grown at either ambient CO_2 (380 µmol C mol-1) or elevated CO_2 (700 µmol C mol-1) and at either 20/16°C (NA) or 5/5°C (CA).

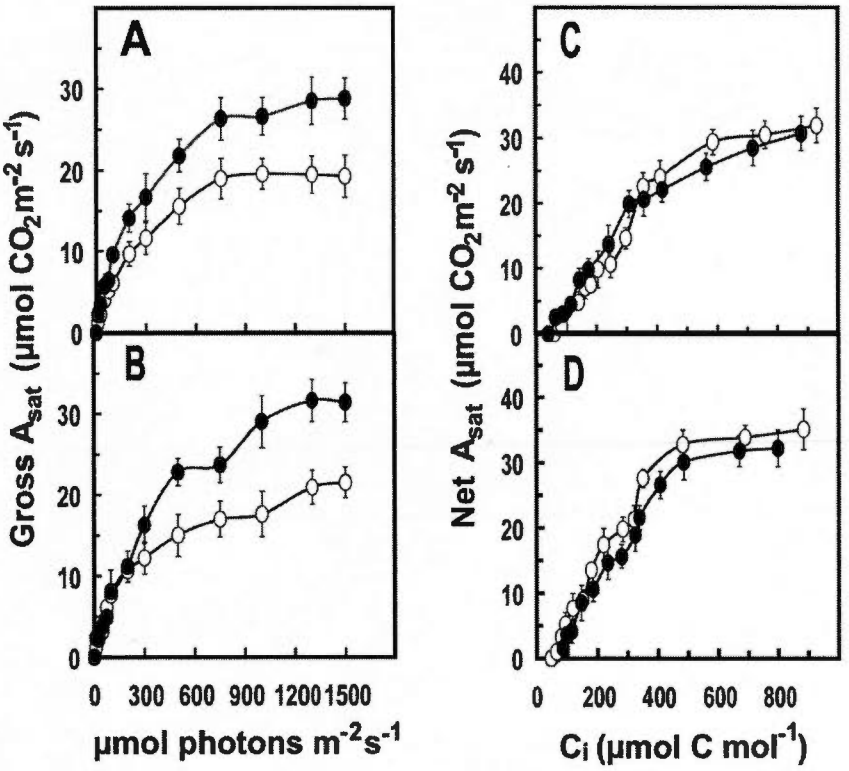

Figure 4.2: Light and CO2 response curves for Norstar wheat grown at ambient or elevated CO2 in non-acclimated (NA) or cold-acclimated (CA) conditions. Light response curves of gross CO_2 assimilation (A, B) and CO_2 response curves of light-saturated net CO_2 assimilation (C, D) for Norstar winter wheat grown at either ambient CO_2 (380 µmol C mol^{-1}, O) or elevated CO_2 (700 µmol C mol^{-1}, ●) and at either 20/16°C (NA; A, C) or 5/5°C (CA; B, D). Measurements were carried out on attached, fully developed third leaves at a measuring temperature of 20°C. Each point represents the mean of nine plants from three different pots. Bars represent SD.

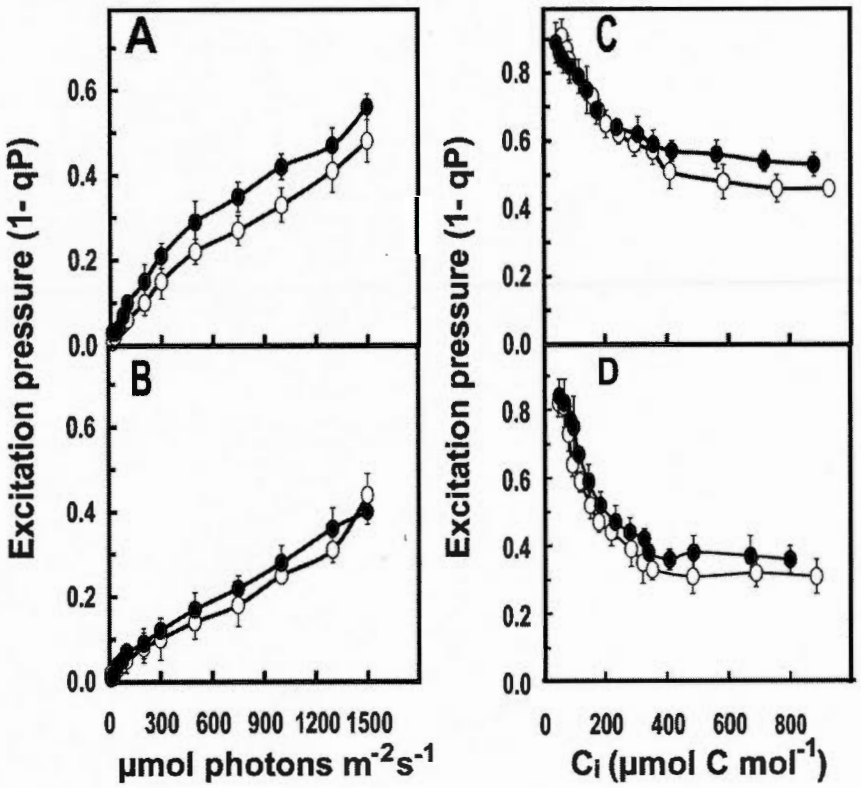

Figure 4.3: Light response curves of excitation pressure (A, B) and CO2 response curves of light-saturated excitation pressure (C, D) for Norstar winter wheat grown at either ambient CO2 (380 µmol C mol-1, O) or elevated CO2 (700 µmol C mol-1, ●) and at either 20/16°C non-acclimated (NA; A, C) or 5/5°C cold-acclimated (CA; B, D). Measurements were carried out on attached, fully developed third leaves at a measuring temperature of 20°C. Each point represents the mean of nine plants from three different pots. Bars represent

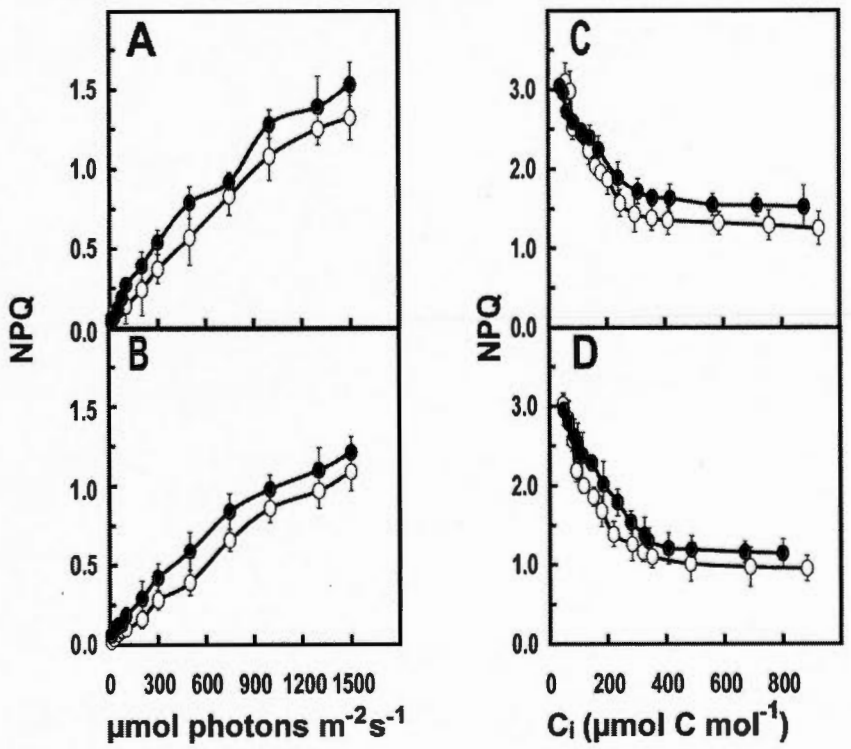

Figure 4.4: Light response curves of non-photochemical quenching of excess energy (NPQ; A, B) and CO2 response curves of light-saturated NPQ (C, D) for Norstar winter wheat grown at either ambient CO2 (380 µmol C mol-1, O) or elevated CO2 (700 µmol C mol-1, ●) and at either 20/16°C non-acclimated (NA; A, C) or 5/5°C cold-acclimated (CA; B, D). Measurements were carried out on attached, fully developed third leaves at a measuring temperature of 20°C. Each point represents the mean of nine plants from three different pots. Bars represent SD.

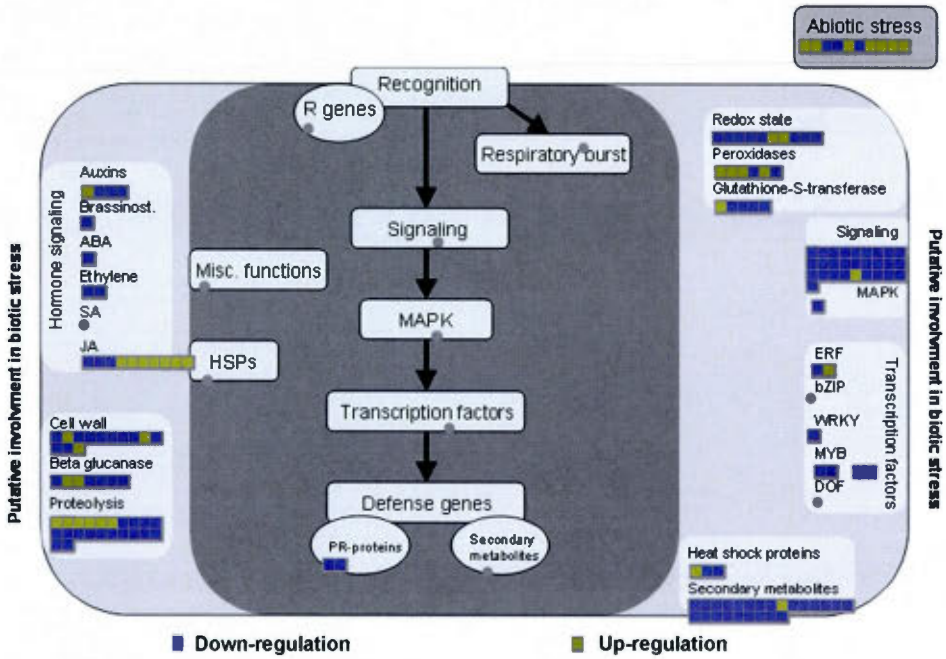

Figure 4.5: MAPMAN overview of the defense-related genes co-ordinately regulated by elevated CO2 in non-acclimated (NA) wheat. Each square represents a gene (blue square is down-regulated and yellow square is up-regulated).The name, category and fold change of the genes are listed in Table S4

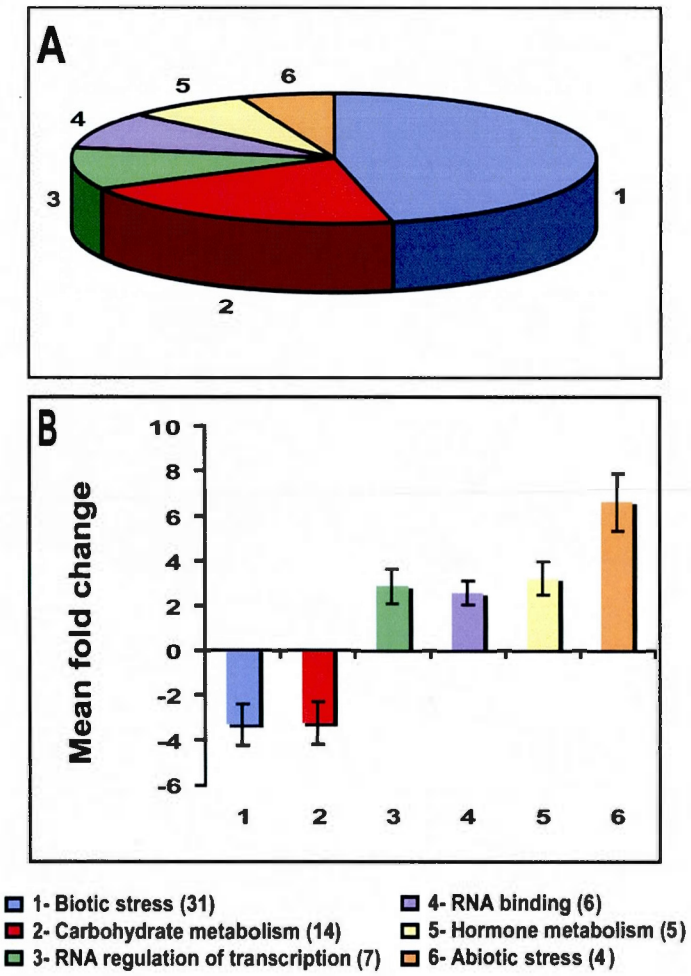

Figure 4.6: MAPMAN functional categories of genes co-ordinately regulated by elevated carbon dioxide in non-acclimated wheat and having quite similar fold change within a category.The genes retained are for those functional groups that exhibited a significantly (Benjamini Hochberg corrected wilcoxon rank sum test; $p \leq 0.05$) different behaviour in terms of expression profiles compared with all the remaining groups during exposure to elevated CO_2. Numbers in parenthesis represent the genes in each category. The name, category and fold change of the genes are listed in Table 3. **A)** Pie chart showing the number of genes within each category.**B)** Mean normalized expression (fold change) of the genes for each functional group retained.

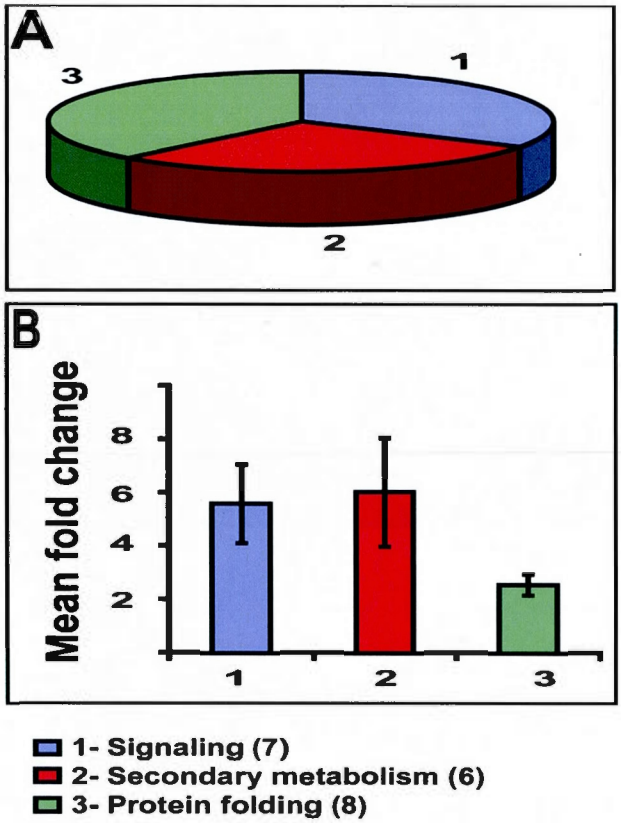

Figure 4.7: MAPMAN functional categories of genes co-ordinately regulated by elevated carbon dioxide in cold-acclimated wheat and having quite similar fold change within a category. The genes retained are for those functional groups that exhibited a significantly (Benjamini Hochberg corrected wilcoxon rank sum test; $p \leq 0.05$) different behaviour in terms of expression profiles compared with all the remaining groups during exposure to elevated CO_2. Numbers in parenthesis represent the genes in each category. The name, category and fold change of the genes are listed in Table 6. **A)** Pie chart showing the number of genes within each category.**B)** Mean normalized expression (fold change) of the genes for each functional group retained.

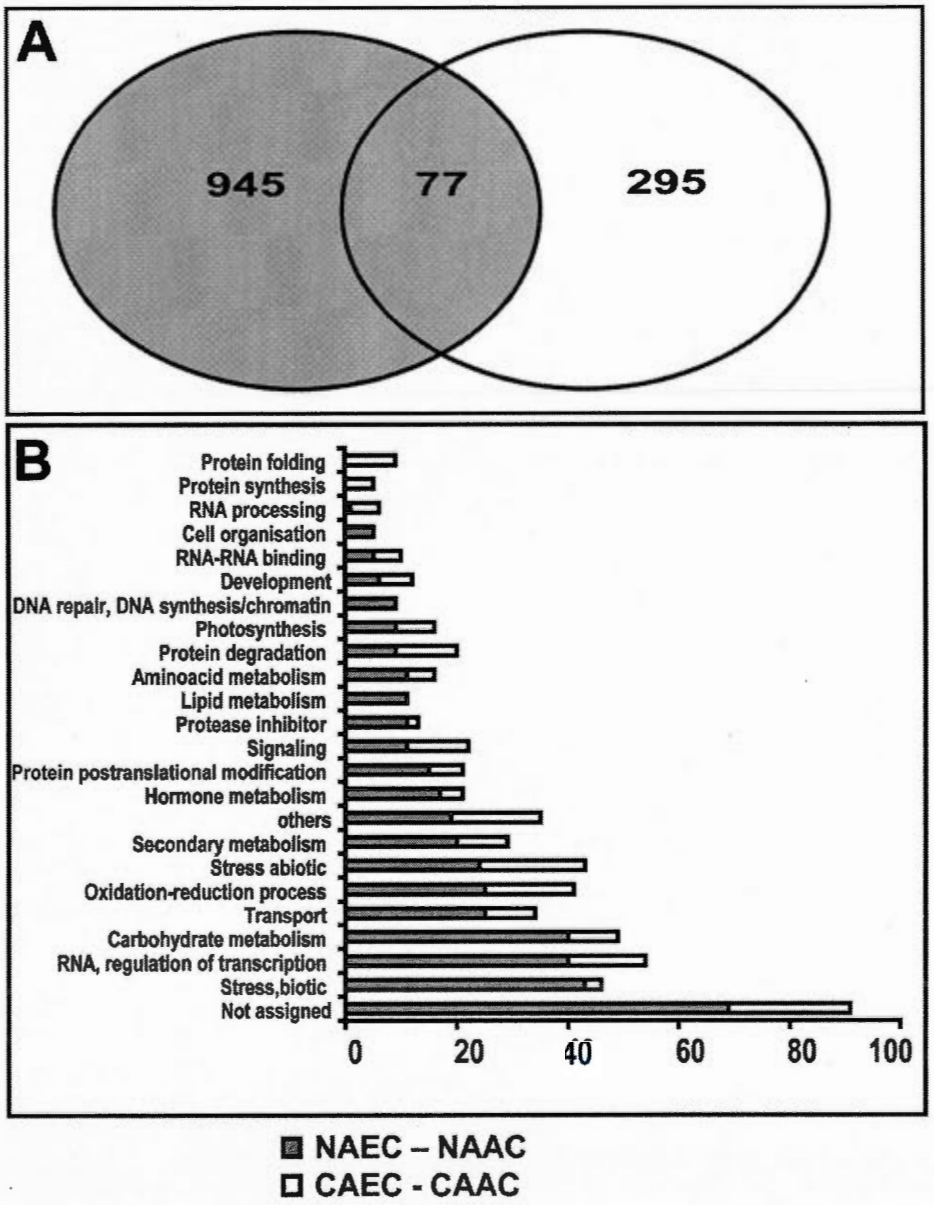

Figure 4.8: Representation of transcript abundance during exposure to elevated CO2 in non-acclimated (NA) and cold-acclimated wheat (CA). (A) Venn diagram giving the numbers of genes specifically and commonly

regulated by elevated CO2 in NA and CA wheat. (B) Comparison of the categorical distribution of genes regulated by elevated CO2 in NA (grey) and CA (white) wheat. NAEC-NAAC corresponds to genes regulated by elevated CO_2 in NA wheat. CAEC-CAAC corresponds to genes regulated by elevated CO_2 in CA wheat.

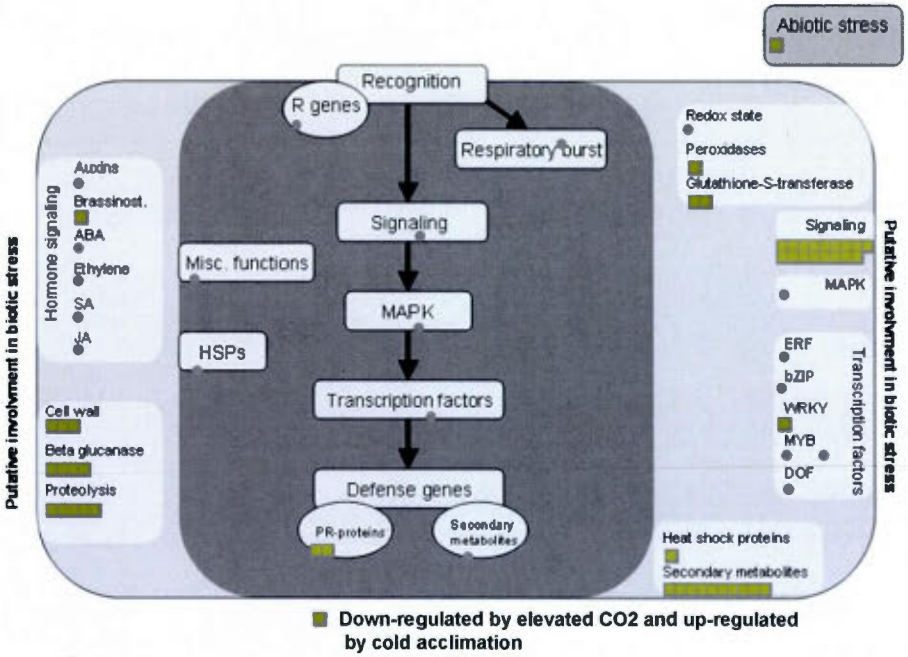

Figure 4.9: MAPMAN overview of 48 defense-related genes down-regulated by elevated CO2 in non-acclimated wheat and up-regulated by cold acclimation at ambient CO2. Each square represents a gene. The list of these genes and their fold change are represented in Table 7.

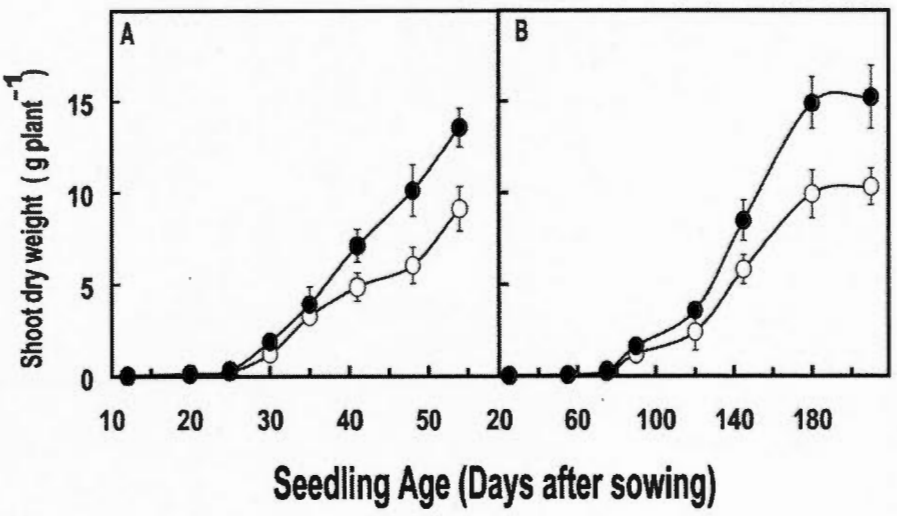

Figure 4.S1: Effects of cold acclimation and elevated CO2 on shoot dry weight of Norstar winter wheat. Plants were grown at either ambient CO2 (380 µmol C mol-1,O) or elevated CO2 (700 µmol C mol-1,●) and at either 20/16°C non-acclimated (NA; A) or 5/5°C cold-acclimated (CA; B). Each point represents the mean of nine plants from three different pots. Bars represent SD.

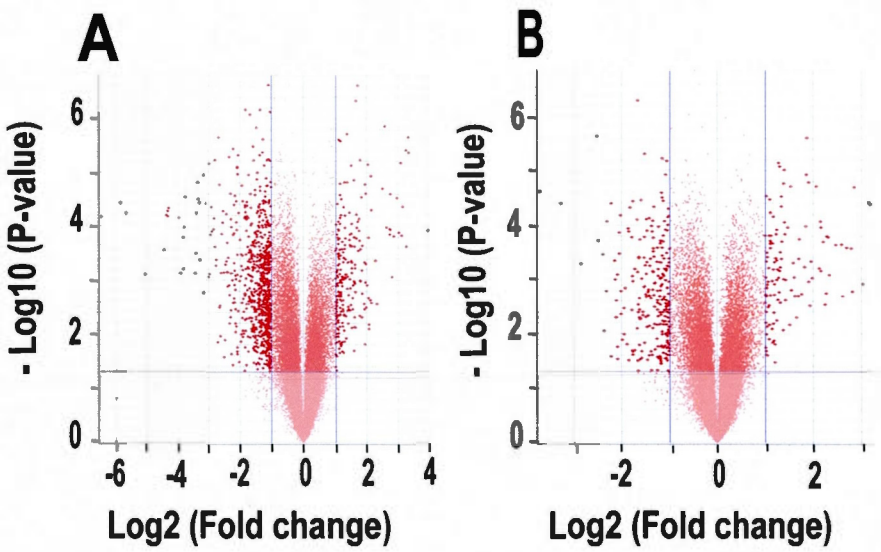

Figure 4.S2: Expression profiling of genes regulated by elevated CO2 in non-acclimated (NA) or cold-acclimated (CA) wheat A. Effect of elevated CO2 in NA wheat B. Effect of elevated CO2 in CA wheat. Volcano plots illustrate logarithmic fold-change expression and p value distribution. Genes that are up or down 2-fold change in expression, with a p value less or equal 0.05, are represented by coloured squares. The name, fold-change and p value of the regulated genes are listed in Tables S2 (NA wheat) and S5 (CA wheat).

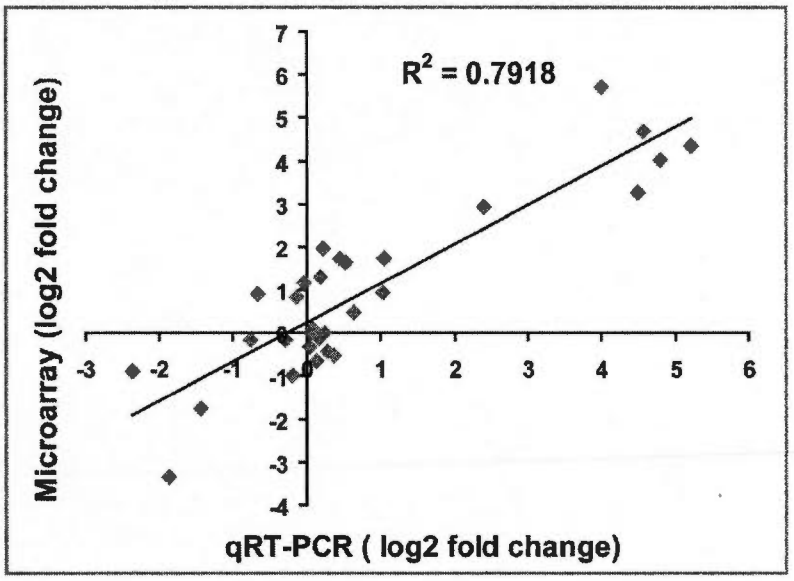

Figure 4.S3: Correlation of qRT-PCR and microarray data. The gene expression (log2 scale) in non-acclimated and cold-acclimated Norstar under ambient CO_2 and elevated CO_2 of the microarray was plotted against the qRT-PCR analyses.

4.7 SUPPORTING INFORMATION

See the CD joined to the thesis

Table 4.S1. Sequences of the primers used for real-time PCR validation

Table 4.S2. Genes regulated by elevated CO_2 in non-acclimated wheat

Table 4.S3. Functional category of the genes regulated by elevated CO_2 in non-acclimated wheat.

Table 4.S4. Genes co-ordinately regulated by elevated CO_2 in non-acclimated wheat and involved in plant defense system against biotic/abiotic stress.

Table 4.S5. Genes regulated by elevated CO_2 in cold-acclimated wheat

Table 4.S6. Functional category of genes regulated by elevated CO_2in cold-acclimated wheat.

Table 4.S7. Common genes regulated by elevated CO_2 in non- and cold-acclimated wheat.

Table 4.S8. Common genes regulated by elevated CO_2 in non-acclimated wheat and cold acclimation at ambient CO_2.

Table 4.S9. Genes regulated by cold acclimation in wheat at ambient CO_2.

4.8 REFERENCES

Adamska I (2004) The Elip family of stress proteins in the thylakoid membranes of pro-and eukaryota. Regulation of photosynthesis 11: 487-505

Adamska I, Ohad I, Kloppstech K (1992) Synthesis of the early light-inducible protein is controlled by blue light and related to light stress. Proc Natl Acad Sci USA 89: 2610-2613

Ahsan N, Lee DG, Kim KH, Alam I, Lee SH, Lee KW, Lee H, Lee BH (2010) Analysis of arsenic stress-induced differentially expressed proteins in rice leaves by two-dimensional gel electrophoresis coupled with mass spectrometry. Chemosphere 78: 224-231

Ainsworth EA, Leakey ADB, Ort DR, Long SP (2008) FACE-ing the facts: inconsistencies and interdependence among field, chamber and modeling studies of elevated [CO_2] impacts on crop yield and food supply. New Phytol 179: 5-9

Ainsworth EA, Long SP (2005) What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. New Phytol 165: 351-371

Ainsworth EA, Rogers A (2007) The response of photosynthesis and stomatal conductance to rising [CO_2]: mechanisms and environmental interactions. Plant Cell Environ 30: 258-270

Ainsworth EA, Rogers A, Vodkin LO, Walter A, Schurr U (2006) The effects of elevated CO_2 concentration on soybean gene expression. An analysis of growing and mature leaves. Plant Physiol 142: 135-147

Albert M, K Jehle A, Lipschis M, Mueller K, Zeng Y, Felix G (2010) Regulation of cell behaviour by plant receptor kinases: pattern recognition receptors as prototypical models. Eur J Cell Biol 89: 200-207

Andersson A, Keskitalo J, Sjodin A, Bhalerao R, Sterky F, Wissel K, Tandre K, Aspeborg H, Moyle R, Ohmiya Y (2004) A transcriptional timetable of autumn senescence. Genome Biol 5: R24

Armstrong AF, Logan DC, Tobin AK, O'Toole P, Atkin OK (2006) Heterogeneity of plant mitochondrial responses underpinning respiratory acclimation to the cold in *Arabidopsis thaliana* leaves. Plant Cell Environ 29: 940-949

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidases in *Beta vulgaris*. Plant Physiol 24: 1-15

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* **415**: 977-983

Atkin O, Tjoelker MG (2003) Thermal acclimation and the dynamic response of plant respiration to temperature. Trends Plant Sci 8: 343-351

Badawi M, Danyluk J, Boucho B, Houde M, Sarhan F (2007) The CBF gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal CBF s. Mol. Genet Genomics 277: 533-554

Badawi M, Reddy YV, Agharbaoui Z, Tominaga Y, Danyluk J, Sarhan F, Houde M (2008) Structure and functional analysis of wheat ICE (inducer of CBF expression) genes. Plant Cell Physiol 49: 1237-1249

Baker NR (2008) Chlorophyll fluorescence: A probe of photosynthesis *in vivo*

Ann Rev Plant Biol 59: 89-113

Benedict C, Skinner JS, Meng R, Chang Y, Bhalerao R, Hüner NPA, Finn CE, Chen TH, Hurry V (2006) The CBF1-dependent low temperature signaling pathway, regulon and increase in freeze tolerance are conserved in Populus spp. Plant Cell Environ. 29: 1259–1272.

Benko-Iseppon AM, Lins Galdino S, Calsa J, Akio Kido E, Tossi A, Carlos Belarmino L, Crovella S (2010) Overview on plant antimicrobial peptides. Curr Prot Pept Sci 11: 181-188

Bernacchi CJ, Leakey ADB, Heady LE, Morgan PB, Dohleman FG, McGrath JM, Gillespie KM, Wittig VE, Rogers A, Long SP, Ort DR (2006) Hourly and seasonal variation in photosynthesis and stomatal conductance of soybean grown at future CO2 and ozone concentrations for 3 years under fully open-air field conditions. Plant Cell Environ 29: 2077-2090

Bertrand A, Prevost D, Bigras FJ, Castonguay Y (2007) Elevated atmospheric CO_2 and strain of rhizobium alter freezing tolerance and cold-induced molecular changes in alfalfa (*Medicago sativa*). Ann Bot 99: 275-284

Bidart-Bouzat MG, Mithen R, Berenbaum MR (2005) Elevated CO_2 influences herbivory-induced defense responses of *Arabidopsis thaliana*. Oecologia 145: 415-424

Bigras F, Bertrand A (2006) Responses of *Picea mariana* to elevated CO_2 concentration during growth, cold hardening and dehardening: phenology, cold tolerance, photosynthesis and growth. Tree Physiol 26: 875-888

Bischof M, Eichmann R, Hückelhoven R (2011) Pathogenesis-associated transcriptional patterns in Triticeae. J Plant Physiol 168: 1618-1328

Bloom AJ, Smart DR, Nguyen DT, Searles PS (2002) Nitrogen assimilation and growth of wheat under elevated carbon dioxide. Proc. Natl. Acad. Sci. USA 99: 1730-1735

Bloom, AJ., Burger M, Asensio JSR, A B. Cousins AB (2010) Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. Science, 328: 899-903

Boese SR, Hüner NPA (1990) Effect of growth temperature and temperature shifts on spinach leaf morphology and photosynthesis. Plant Physiol 94: 1830-1836

Buchanan-Wollaston V, Ainsworth C (1997) Leaf senescence in Brassica napus: cloning of senescence-related genes by subtractive hybridisation. Plant Molecular Biology 33: 821–834.

Casson SA, Franklin KA, Gray JE, Grierson CS, Whitelam GC, Hetherington AM (2009) Phytochrome B and PIF4 regulate stomatal development in response to light quantity. Curr Biol 19: 229-234

Casteel CL, O'neil BF, Zavala JA, Bilgin DD, Berenbaum MR, Delucia E (2008) Transcriptional profiling reveals elevated CO_2 and elevated O_3 alter resistance of soybean (*Glycine max*) to Japanese beetles (*Popillia japonica*). Plant Cell Environ. 31: 419-434

Chen G-Y, Yong Z-H, Liao Y, Zhang D-Y, Chen Y, Zhang H-B, Chen J, Zhu J-G, Xu D-Q (2005) Photosynthetic acclimation in rice leaves to free-air CO_2 enrichment related to both ribulose-1,5-bisphosphate carboxylation limitation and ribulose-1,5-bisphosphate regeneration limitation. Plant Cell Physiol 46: 1036-1045

Dahal K, Gadapati W, Savitch LV, Singh J, Hüner NPA (2012a) Cold acclimation and *BnCBF17*-overexpression enhance photosynthetic performance and

energy conversion efficiency during long-term growth of *Brassica napus* under elevated CO_2 conditions. Planta, *in press* (doi 10.1007/s00425-012-1710-2)

Dahal K, Kane K, Gadapati W, Webb E, Savitch LV, Singh J, Sharma P, Sarhan F, Longstaffe FJ, Grodzinski B, Hüner NPA (2012b) The effects of phenotypic plasticity on photosynthetic performance in winter rye, winter wheat and *Brassica napus*. Physiol Plant 144: 169-188

Dahal K, Kane K, Sarhan F, Grodzinski B, Hüner NPA (2012c) Cold acclimation inhibits CO_2-dependent stimulation of photosynthesis in spring wheat and spring rye. Botany 90: 433-444

Danyluk J, Perron A, Houde M, Limin A, Fowler B, Benhamou N, Sarhan F (1998) Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell 10: 623-638

Delucia EH, Sasek TW, Strain BR (1985) Photosynthetic inhibition after long-term exposure to elevated levels of atmospheric carbon dioxide. Photosyn Res. 7: 175-184

De Lucia E, Nabity P, Zavala J, Berenbaum M (2012) Climate change: resetting plant-insect interactions. 10.1104/pp.112.204750. Plant Physiol Online

Demmig-Adams B, Adams III WW (1992) Photoprotection and other responses of plants to high light stress. Ann Rev Plant Physiol Plant Mol Biol 43: 599-626

Di Toppi LS, Marabottini R, Badiani M, Raschi A (2002) Antioxidant status in herbaceous plants growing under elevated CO_2 in mini-FACE rings. J. Plant Physiol. 159: 1005-1013

Drake BG, Gonzàlez-Meler MA, Long SP (1997) More efficient plants: a consequence of rising atmospheric CO_2? Annu Rev Plant Mol Biol 48: 609-639

Eckey C, Korell M, Leib K, Biedenkopf D, Jansen C, Langen G, Kogel KH (2004) Identification of powdery mildewinduced barley genes by cDNA-AFLP: functional assessment of an early expressed MAP kinase. Plant Mol Biol 55: 1–15

Ensminger I, Busch F, Hüner NPA (2006) Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol Plant 126:28–44

Farquhar GD (1997) Carbon dioxide and vegetation. Science 278: 1411

Ferrer JL, Jez JM, Bowman ME, Dixon RA, Noel JP (1999) Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat Struct Biol 6: 775-784

Foyer C, Furbank R, Harbinson J, Horton P (1990) The mechanisms contributing to photosynthetic control of electron transport by carbon assimilation in leaves. Photosynth Res 25: 83-100

Fukayama H, Fukuda T, Masumoto C, Taniguchi Y, Sakai H, Cheng W, Hasegawa T, Miyao M (2009) Rice plant response to long term CO_2 enrichment: Gene expression profiling. Plant Sci. 177: 203-210

Gillespie KM, Rogers A, Ainsworth EA (2011) Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (*Glycine max*). J Exp Bot 62: 2667-2678

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD,Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiology 124: 1854–1865

Gjetting T, Hagedorn PH, Schweizer P, Thordal-Christensen H, Carver TL, Lyngkjaer MF (2007) Single-cell transcript profiling of barley attacked by the powdery mildew fungus. Mol Plant-Microbe Interact 20: 235-246

Gorlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessmann H (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8: 629-643

Gorsuch PA, Pandey S, Atkin OK (2010a) Temporal heterogeneity of cold acclimation phenotypes in *Arabidopsis* leaves. Plant Cell Environ 33: 244-258

Gorsuch PA, Pandey S, Atkin OK (2010b) Thermal de-acclimation: how permanent are leaf phenotypes when cold-acclimated plants experience warming? Plant Cell Environ 33: 1124-1137

Griffith M, Yaish MWF (2004) Antifreeze proteins in overwintering plants: a tale of two activities. Trends Plant Sci. 9: 399-405

Gray GR, Chauvin LP, Sarhan F, Huner NPA (1997) Cold acclimation and freezing tolerance. A complex interaction of light and temperature. Plant Physiol 114: 467-474

Guy CL (1990) Cold acclimation and freezing tolerance: role of protein metabolism. Ann Rev Plant Physiol Plant Mol Biol 41: 187-223

Harley PC, Sharkey TD (1991) An improved model of C3 photosynthesis at high CO_2: reversed O_2 sensitivity explained by lack of glycerate reentry into the chloroplast. Photosynth Res 27:169–178

Heinze B (2007) A database of PCR primers for the chloroplast genomes of higher plants. Plant Methods 3: 4

Hetherington AM, Woodward FI (2003) The role of stomata in sensing and driving environmental change. Nature 424: 901-908

Higgins SI, Scheiter S (2012) Atmospheric CO2 forces abrupt vegetation shifts locally, but not globally. Nature 488: 209-212

Hiilovaara-Teijo M, Hannukkala A, Griffith M, Yu XM, Pihakaski-Maunsbach K (1999) Snow-mold-induced apoplastic proteins in winter rye leaves lack antifreeze activity. Plant Physiol 121: 665-673

Houde M, Dhindsa RS, Sarhan F (1992) A molecular marker to select for freezing tolerance in Gramineae. Mol Gen Genet 234: 43-48

Houghton JT (2001) Climate change 2001: the scientific basis, Vol 881. Cambridge University Press Cambridge, UK

Hüner NPA, Palta JP, Li PH, Carter JV (1981) Comparison of the structure and function of ribulose bisphosphate carboxylase-oxygenase from a cold-hardy and nonhardy potato species. Can J Biochem. 59:280-9

Hüner NPA (1985) Morphological, anatomical and molecular consequences of growth and development at low temperature in *Secale cereale* L. cv Puma. Amer J Bot 73: 1290-1306

Hüner NPA, Öquist G, Hurry VM, Krol M, Falk S, Griffith M (1993) Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. Photosynth Res. 37: 19-39

Hüner NPA, Öquist G, Sarhan F (1998) Energy balance and acclimation to light and cold. Trends Plant Sci 3: 224-230

Hurry VM, Krol M, Öquist G, Hüner NPA (1992) Effect of long-term photoinhibition on growth and photosynthesis of cold-hardened spring and winter wheat. Planta 188: 369-375

Hurry VM, Malmberg G, Gardeström P, Öquist G (1994) Effects of a short-term shift to low temperature and of long-term cold hardening on photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase and sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). Plant Physiol 106: 983-990

Hurry V, Tobiaeson M, Krömer S, Gardeström P, Öquist G (1995) Mitochondria contribute to increased photosynthetic capacity of leaves of winter rye (*Secale cereale* L.) following cold-hardening. Plant Cell Environ 18: 69-76

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) *Arabidopsis CBF1* overexpression induces COR genes and enhances freezing tolerance. Science 280: 104-106

Jiang CJ, Shimono M, Maeda S, Inoue H, Mori M, Hasegawa M, Sugano S, Takatsuji H (2009) Suppression of the rice fatty-acid desaturase gene OsSSI2 enhances resistance to blast and leaf blight diseases in rice. Mol Plant Microbe Interact 22:820-829

Krause GH (1988) Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. Physiol Plant 74: 566-574

Krol M, Griffith M, Hüner NPA (1984) An appropriate physiological control for environmental temperature studies: comparative growth kinetics of winter rye. Can J Bot 62: 1062-1068

Kuwabara C, Imai R (2009) Molecular basis of disease resistance acquired through cold acclimation in overwintering plants. J Plant Biol 52: 19-26

Lee C-M, Thomashow MF (2012) Photoperiodic regulation of the C-repeat binding factor (CBF) cold acclimation pathway and freezing tolerance in *Arabidopsis thaliana* Proc Natl Acad Sci USA 109: 15054-15059

Lee TD, Tjoelker MG, Ellsworth DS, Reich PB (2001) Leaf gas exchange responses of 13 prairie grassland species to elevated CO_2 and increased nitrogen supply. New Phytol 150: 405-418

Li P, Ainsworth EA, Leakey AD, Ulanov A, Lozovaya V, Ort DR, Bohnert HJ (2008) Arabidopsis transcript and metabolite profiles: ecotype-specific responses to open-air elevated (CO_2). Plant Cell Environ 31: 1673-1687

Li Z, Wakao S, Fischer BB, Niyogi KK (2009) Sensing and responding to excess light.

Ann Rev Plant Biol 60: 239-260

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods 25: 402-408

Long SP, Ainsworth EA, Rogers A, Ort DR (2004) Rising atmospheric carbon dioxide: plants FACE the future. Annu Rev Plant Biol 55: 591-628

Marocco A, Wissenbach M, Becker D, Paz-Ares J, Saedler H, Salamini F, Rohde W (1989) Multiple genes are transcribed in *Hordeum vulgare* and *Zea mays* that carry the DNA binding domain of the myb oncoproteins. Mol Gen Genet 216: 183-187

Martin M, Gavazov K, Koerner C, Haettenschwiler S, Rixen C (2010) Reduced early growing season freezing resistance in alpine treeline plants under elevated atmospheric CO_2. Global Change Biol. 16: 1057-1070

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue: II. Inhibition of fungal growth by combinations of chitinase and -1, 3-glucanase. Plant Physiol 88: 936-942

Maurel C, Verdoucq L, Luu D-T, Santoni V (2008) Plant aquaporins: membrane channels with multiple integrated functions. Annu Rev Plant Biol 59: 595-624

Monte E, Al-Sady B, Leivar P, Quail PH (2007) Out of the dark: how the PIFs are unmasking a dual temporal mechanism of phytochrome signalling. J Exp Bot 58: 3125-3133

Moore B, Cheng SH, Rice J, Seemann J (1998) Sucrose cycling, Rubisco expression, and prediction of photosynthetic acclimation to elevated atmospheric CO_2. Plant Cell Environ 21: 905-915

Morgan JA, LeCain DR, Pendall E, Blumenthal DM, Kimball BA, Carrillo Y, Williams DG, Heisler-White J, Dijkstra FA, West M (2011) C4 grasses

prosper as carbon dioxide eliminates desiccation in warmed semi-arid grassland. Nature 476: 202-205

Muthukrishnan S, Liang GH, Trick HN, Gill BS (2001) Pathogenesis-related proteins and their genes in cereals. Plant Cell Tiss Org Cult 64: 93-114

Nakamura T, Ohta M, Sugiura M, Sugita M (2001) Chloroplast ribonucleoproteins function as a stabilizing factor of ribosome-free mRNAs in the stroma. J Biol Chem 276: 147-152

NDong C, Danyluk J, Wilson KE, Pocock T, Huner NPA, Sarhan F (2002) Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. Plant Physiol 129: 1368-1381

Ogo Y, Itai RN, Nakanishi H, Inoue H, Kobayashi T, Suzuki M, Takahashi M, Mori S, Nishizawa NK (2006) Isolation and characterization of IRO2, a novel iron-regulated bHLH transcription factor in graminaceous plants. J Exp Bot 57: 2867-2878

Öquist G, Hurry VM, Hüner NPA (1993) Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. Plant Physiol 101: 245

Öquist, G, Hüner NPA (2003) Photosynthesis of overwintering evergreen plants. Ann Rev Plant Biol 54: 329-355

Ort DR (2001) When there is too much light. Plant Physiol 125: 29-32

Pocock TH, Hurry V, Savitch LV, Hüner N (2001) Susceptibility to low temperature photoinhibition and the acquisition of freezing tolerance in winter and spring wheat: The role of growth temperature and irradiance. Physiol Plant 113: 499-506

Polle A, Eiblmeier M, Sheppard L, Murray M (1997) Responses of antioxidative enzymes to elevated CO_2 in leaves of beech (*Fagus sylvatica L.*) seedlings grown under a range of nutrient regimes. Plant Cell Environ 20: 1317-1321

Ponce-Valadez M, Fellman SM, Giovannoni J, Gan SS, Watkins CB (2009) Differential fruit gene expression in two strawberry cultivars in response to elevated CO_2 during storage revealed by a heterologous fruit microarray approach. Postharvest Biol Technol 51: 131-140

Pötter E, Kloppstech K (1993) Effects of light stress on the expression of early light-inducible proteins in barley. Eur J Biochem 214: 779-786

Pritchard SG, Ju Z, Santen VE, QiuD J, Weaver DB, Prior SA, Rogers HH (2000) The influence of elevated CO_2 on the activities of antioxidative enzymes in two soybean genotypes. Aust J Plant Physiol 27: 1061-1068

Quirino BF, Noh YS, Himelblau E, Amasino RM (2000) Molecular aspects of leaf senescence. Trends Plant Sci 5: 278-282

Rao MV, Hale BA, Ormrod DP (1995) Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide (role of antioxidant enzymes). Plant Physiol 109: 421-432

Rochaix J (1995) *Chlamydomonas reinhardtii* as the photosynthetic yeast. Annu Rev Genet 29: 209-230

Romeis T (2001) Protein kinases in the plant defence response. Curr Opin Plant Biol 4: 407 -414

Rosso D, Bode R, Li W, Krol M, Saccon D, Wang S, Schillaci LA, Rodermel SR, Maxwell DP, Huner NPA (2009) Photosynthetic redox imbalance governs leaf sectoring in the *Arabidopsis thaliana* variegation mutants immutans, spotty, var1, and var2. Plant Cell 21: 3473-3492

Sarhan F, Perras M (1987) Accumulation of a high molecular weight protein during cold hardening of wheat (*Triticum aestivum L.*). Plant Cell Physiol. 28: 1173

Savitch LV, Allard G, Seki M, Robert LS, Tinker NA, Hüner NPA, Shinozaki K, Singh J (2005) The effect of over-expression of two Brassica *CBF/DREB1*-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. Plant Cell Physiol. 46: 1525-1539

Savitch LV, Barker-Astrom J, Ivanov A G, Hurry V, Oquist G, Hüner N P A, Gardestrom P (2001) Cold acclimation of Arabidopsis thaliana results in incomplete recovery of photosynthetic capacity which is associated with an increased reduction of the chloroplast stroma. Planta 214: 295-301

Schlumbaum A, Mauch F, Vögeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365-367

Schwanz P, Polle A (2001) Growth under elevated CO_2 ameliorates defenses against photo-oxidative stress in poplar (*Populus alba x tremula*). Environ Exp Bot 45: 43-53

Shen QH, Saijo Y, Mauch S, Biskup C, Bieri S, Keller B, Seki H, Ulker B, Somssich IE, Schulze-Lefert P (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science 315 1098–1103

Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (2007) Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change, 2007. IPCC. Cambridge University Press, Cambridge, UK and New York, NY, USA

Stafford HA, Ibrahim RK (1992) Phenolic metabolism in plants. Plenum Press New York, London 432

Stitt M, Hurry V (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Curr Opin Plant Biol 5: 199-206

Stitt M, Quick WP (1989) Photosynthetic carbon partitioning: its regulation and possibilities for manipulation. Plant Physiol 77: 633-641

Strand A, Hurry V, Henkes S, Hüner NPA, Gustafsson P, Gardestrom P, Stitt M (1999) Acclimation of Arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol 119: 1387-1397

Strand A, Foyer CH, Gustafsson P, Gardestrom P, Hurry V (2003) Altering flux through the sucrose biosynthesis pathway in transgenic *Arabidopsis thaliana* modifies photosynthetic acclimation at low temperatures and the development of freezing tolerance. Plant Cell Environ 26: 523-535

Tao Z, Liu H, Qiu D, Zhou Y, Li X, Xu C, Wang S (2009) A pair of allelic WRKY genes play opposite roles in Rice-Bacteria interactions. Plant Physiol. 151: 936-948

Taylor G, Street NR, Tricker PJ, Sjödin A, Graham L, Skogström O, Calfapietra C, Scarascia-Mugnozza G, Jansson S (2005) The transcriptome of Populus in elevated CO_2. New Phytol. 167: 143-154

Tcherkez GG, Farquhar GD, Andrews TJ (2006) Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. Proc Natl Acad Sci U S A 103: 7246-7251

Theocharis A, Clemente C, Barka EA (2012) Physiological and molecular changes in plants grown at low temperatures. Planta 235: 1091-1105

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol 118: 1-8

Thomma BPHJ, Penninckx IAMA, Cammue B, Broekaert WF (2001) The complexity of disease signaling in Arabidopsis. Curr Opin Immunol 13: 63-68

Tonkaz T, Dogan E, Kocyigit R (2010) Impact of temperature change and elevated carbon dioxide on winter wheat (*Triticum aestivum L.*) grown under semi-arid conditions. Bulg J Agric Sci 16: 565-575

Tremblay K, Ouellet F, Fournier J, Danyluk J, Sarhan F (2005) Molecular characterization and origin of novel bipartite cold-regulated ice recrystallization inhibition proteins from cereals. Plant Cell Physiol 46: 884

Van Buskirk HA, Thomashow MF (2006) Arabidopsis transcription factors regulating cold acclimation. Physiol Plant 126: 72-80

Van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol 44: 135-162

Vigers AJ, Wiedemann S, Roberts WK, Legrand M, Selitrennikoff CP, Fritig B (1992) Thaumatin-like pathogenesis-related proteins are antifungal. Plant Sci 83: 155-161

Wang X, Liu W, Chen X, Tang C, Dong Y, Ma J, Huang X, Wei G, Han Q, Huang L (2010) Differential gene expression in incompatible interaction between wheat and stripe rust fungus revealed by cDNA-AFLP and comparison to compatible interaction. BMC Plant Biol 10: 9

Wissenbach M, Überlacker B, Vogt F, Becker D, Salamini F, Rohde W (1993) Myb genes from Hordeum vulgare: tissue-specific expression of chimeric Myb promoter/Gus genes in transgenic tobacco. Plant J 4: 411-422

Woodward F, Lake J, Quick W (2002) Stomatal development and CO_2: ecological consequences. New Phytol 153: 477-484

Wu G, Chen FJ, Ge F, Xiao N-W (2011) Impacts of elevated CO_2 on expression of plant defensive compounds in Bt-transgenic cotton in response to infestation by cotton bollworm. Agric For Entomol 13: 77-82

Yeh S, Moffatt BA, Griffith M, Xiong F, Yang DSC, Wiseman SB, Sarhan F, Danyluk J, Xue YQ, Hew CL (2000) Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiol 124: 1251-1264

Zavala JA, Casteel CL, DeLucia EH, Berenbaum MR (2008) Anthropogenic increase in carbon dioxide compromises plant defense against insects. Proc Natl Acad Sci USA 105: 5129-5133.

Zhang DY, Chen GY, Chen J, Yong ZH, Zhu JG, Xu DQ (2009) Photosynthetic acclimation to CO_2 enrichment related to ribulose-1,5-bisphosphate carboxylation limitation in wheat. Photosynthetica 47: 152-154

Zhou JM, Gold ND, Martin VJJ, Wollenweber E, Ibrahim RK (2006) Sequential O-methylation of tricetin by a single gene product in wheat. Biochim Biophys Acta Gen Subj 1760: 1115-1124

CONCLUSION

Les résultats présentés dans cette thèse de doctorat montrent que les céréales tolérantes au gel lorsqu'acclimatées au froid ont la capacité de modifier leur réponse physiologique et génétique pour compenser les effets d'une élévation importante de la concentration en CO_2.

Les analyses biochimiques et moléculaires du chapitre II montrent que le blé et le seigle d'hiver (cv Norstar et cv Musketeer) acclimatées au froid contrairement aux variétés printanières (Katepwa et SR4A) augmentent leur efficacité et taux de transfert d'électrons photosynthétiques, diminuent la pression d'excitation au niveau de leur photosystème II et augmentent la dissipation de l'énergie sous forme de chaleur, démontrant ainsi une meilleure performance face à l'inhibition photosynthétique associé au froid. La surexpression de BNCBF17 chez *Brassica napus* indiquent que les gènes CBFs/DREBs semblent réguler la tolérance au gel et gouvernent l'architecture des plantes, l'anatomie des feuilles, la performance photosynthétique et l'efficacité de l'utilisation de l'eau.

Par la suite, sachant que les variétés de printemps acclimatés au froid n'arrivent pas à empêcher l'inhibition de leur assimilation de CO_2, nous avons voulu savoir si l'augmentation de CO_2 pouvait compenser cette inhibition photosynthétique associée à l'acclimatation au froid. Les résultats de cette étude dans le chapitre 3 ont montré que les variétés printanières acclimatées au froid n'arrivent pas à récupérer de l'inhibition de l'assimilation de CO_2 induite par le froid suite à une exposition courte aux concentrations élevées de CO_2. A l'opposé les variétés hivernales de blé et de

seigle acclimaté au froid présentent une hausse de 12 et de 37% de leur capacité photosynthétique suite à une exposition courte aux concentrations élevées de CO_2 relativement aux plantes non acclimatées.

Finalement, sachant que l'acclimatation au froid peut maintenir la stimulation à court terme de la photosynthèse par les concentrations élevées de CO_2, nous avons cherché à savoir si cette stimulation est préservée durant une longue exposition aux concentrations élevées de CO_2. Dans le dernier chapitre, nous avons montré que le blé d'hiver peut maintenir la stimulation induite par les concentrations élevées de CO_2 durant une longue exposition aux concentrations élevées de CO_2. Plusieurs plantes de types C3 sont sujettes à la photoinihbition engendrée par l'accumulation des hydrates de carbone lorsqu'elles sont exposées à des concentrations élevées de CO_2 durant une longue période de temps. Chez le blé d'hiver Norstar, l'exposition prolongée à des concentrations élevées de CO_2 n'a pas inhibée la photosynthèse, se traduisant en une plus grande augmentation de sa biomasse comparativement aux concentrations ambiantes de CO_2. Néanmoins, la hausse de biomasse associée aux concentrations élevées de CO_2 est plus importante chez le blé d'hiver acclimaté comparativement au non acclimaté. De plus, la conductance et la densité au niveau des stomates diminuent avec les concentrations élevées de CO_2 ainsi que durant l'acclimatation au froid, ce qui permet au blé de diminuer son évapotranspiration et d'augmenter son éfficacité d'utilisation de l'eau. L'analyse du transcriptome du blé acclimaté et non acclimaté au froid en présence des concentrations élevées de CO_2 a permis d'identifier les facteurs génétiques impliqués dans cette hausse de performance photosynthétique. Cette hausse de performance photosynthétique du blé d'hiver Norstar acclimaté au froid est attribuable à sa capacité d'induire des gènes impliqués dans la stabilité et la protection du cholroplaste. Par ailleurs, l'exposition à un stress préalable peut conférer ou diminuer la résistance face à un autre stress. Nos résultats ont démontré que l'acclimatation au froid peut conférer un avantage pour la

croissance du blé en concentrations élevées de CO_2. Chez le blé non acclimaté qui pousse à température de 20°C, les concentrations élevées de CO_2 ont entrainé un affaiblissement du système de défense des plantes face aux stress biotiques comme le démontre la forte répression des gènes de défense aux pathogènes. Chez les plantes acclimatées au froid on assiste à un renforcement du système de protection aux plantes face aux stress biotiques. Bien que l'acclimatation au froid solidifie le système de défense des plantes, il n'en demeure pas moins que le blé d'hiver reste vulnérable à l'attaque par les pathogènes comme le démontrent les fortes répressions des gènes thionins impliqués dans l'immunité systémique acquise des plantes.

Les conséquences économiques et sociales de l'interaction des plantes avec les pathogènes et les insectes sont énormes et seront accentuées par le réchauffement climatique dû à l'augmentation des gaz à effet de serre. Les pertes engendrées par les bactéries virus et champignons peuvent être contrées par la sélection de cultivars tolérants ayant la capacité de maintenir leur rendement en présence de ces pathogènes. L'utilisation de rhizobactéries ayant la capacité de renforcer le sytème immunitaire et la croissance des plantes sont des bonnes approches pour limiter les dommages reliés aux pathogènes.

Perspectives

Nos résultats ont montré que le système de défense des plantes face aux pathogènes est réprimé par les concentrations élevées de CO_2 chez le blé d'hiver Norstar, tandis que l'acclimatation au froid permettait d'induire les gènes de défense. Ces résultats suggèrent que le blé acclimaté au froid sera plus résistant à l'attaque par un pathogène. Cette hypothèse pourrait être vérifiée en infectant des plants de blé non

acclimaté et acclimaté avec un pathogène tel que Fusarium en présence des concentrations élevées de CO_2.

APPENDICE A

ARTICLE IV: DAPHNETIN METHYLATION STABILIZES THE ACTIVITY OF PHOSPHORIBULOKINASE IN WHEAT DURING COLD ACCLIMATION

Khalil Kane, Amira Moheb, Yukihara Fukushi, René Roy, Norman P.A Hüner, Ragai K. Ibrahim and Fathey Sarhan

Article publié dans Biochem Cell Biol. 2012 Oct; 90(5) : 657-666

Contribution

Dans cet article qui a été publié dans la revue Biochemistry and cell Biology. J'ai réalisé les expériences de purification de la phosphoribulokinase et de son inhibition par la daphnetine. Tous les auteurs ont participé dans la rédaction du manuscrit.

A.1 RÉSUMÉ

La méthylation de la daphnétine (7,8-dihydroxycoumarin) et de son dérivé 8-méthyle est catalysée par une O-méthyltransférase du blé (*Triticum aestivum L.*) (TaOMT1). Cette enzyme est régulée par le froid et la pression d'excitation du photosystème II (état redox du plastide). Nous examinons ici la signification biologique de cette méthylation et son rôle potentiel dans la modulation de l'activité des kinases du blé. Afin d'identifier les kinases qui peuvent possiblement interagir avec la daphétine du blé, un extrait protéique soluble des parties aériennes de blé acclimaté au froid a été purifié par une séparation sur DEAE-cellulose et une chromatographie d'affinité sur une colonne EAH-sépharose couplée à un dérivé de la daphnétine (acide 7,8 dihydroxy-4-coumarin acétique). Une analyse en spectrométrie de masse a indiqué que la phosphoribulokinase du blé (TaPRK) est la principale kinase qui lie la daphnétine. Cette TaPRK joue un rôle important en régulant le flux de carbone à travers le cycle de Calvin en catalysant l'étape finale de régénération du ribulose 1,5-biphosphate à partir du ribulose 5-phosphate (Ru5P) et l'ATP. Les activités de la TaPRK, endogène ou recombinante, sont inhibées par la daphnétine de façon spécifique et dépendante de la concentration, mais pas par son dérivé mono-méthylé (7-méthyl, 8-hydroxy- coumarin). De plus, l'analyse en HPLC-MS d'extraits de blé révèle que le 7,8 diméthylhydroxycoumarin est plus abondant que son dérivé mono-méthylé. Les résultats montrent aussi que l'acclimatation au froid ne modifie pas le niveau d'ARNm de TaPRK ni son activité enzymatique, faisant en sorte que la génération stable de ribulose 1,5-biphosphate est assurée.

Mots clés : Phosphoribulokinase, O-méthylation, coumarin, acclimatation au froid, Blé

A.2 ABSTRACT

The methylation of daphnetin (7,8-dihydroxycoumarin) to its 8-methyl derivative is catalyzed by a wheat (Triti- cum aestivum L.) O-methyltransferase (TaOMT1). This enzyme is regulated by cold and photosystem II excitation pressure (plastid redox state). Here, we investigated the biological significance of this methylation and its potential role in modulating the activity of kinases in wheat. To identify the potential kinases that may interact with daphnetin in wheat, the soluble protein extract from aerial parts of cold-acclimated wheat was purified by DEAE-cellulose separation and affinity chromatography on a daphnetin derivative (7,8-dihydroxy-4-coumarin acetic acid)-EAH sepharose column. Mass spectrometric analysis indicated that wheat phosphoribulokinase (TaPRK) is the major kinase that binds to daphnetin. This TaPRK plays an important role in regulating the flow of carbon through the Calvin cycle, by catalyzing the final step in the regeneration of ribulose 1,5-bisphosphate from ribulose-5-phosphate (Ru5P) and ATP. The activities of TaPRK, endogenous or recombinant, are inhibited by daphnetin in a specific and dose-dependent manner, but not by its monomethyl derivative (7-methyl, 8-hydroxy- coumarin). Furthermore, HPLC-MS analysis of wheat extracts reveals that 7,8-dimethoxycoumarin is more abundant than its monomethyl derivative. The results also show that cold acclimation does not alter the level of TaPRK mRNA or its enzyme activity, and thus ensures the stable generation of ribulose 1,5-biphosphate.

Key words: phosphoribulokinase, O-Methylation, coumarin, cold acclimation, wheat.

A.3 INTRODUCTION

Low temperature (LT) is one of the most common environmental factors influencing growth, development, and survival of plants. Winter hardy plants are able to tolerate extreme subzero temperatures. This tolerance is not constitutive, and plants require a period of exposure to low, nonfreezing temperature to acquire freezing tolerance (FT), a process called cold acclimation (CA). During this process, plants accumulate several metabolites and proteins that are required for the development of FT (Allard *et al.,* 1998; Houde *et al.,* 1992; Winfield *et al.,* 2010; Winkel-Shirley 2002). Investigation of plant responses to LT is of critical importance to understand how plants cope with chilling and freezing stresses that may potentially lead to the improvement of crop productivity and quality.

In response to various abiotic (low or high temperature, salt, drought) and biotic (fungal elicitors, infection by micro- organisms) stresses, plant proteins can be subjected to a variety of modifications such as acetylation, phosphorylation, and methylation that allow the expression of novel functions. It is well known that the protein phosphorylation mediated pathway, governed by the complementary activities of kinases and phosphatases, plays an important role in the LT signal transduction (Stone and Walker 1995; Vazquez-Tello *et al.,* 1998; Vlad *et al.,* 2008). Functional genomics analysis has been used as a powerful approach for understanding the regulatory role that kinases play in signaling during plant development and survival (Chevalier *et al.,* 2005; Colcombet *et al.,* 2005; Wang *et al.,* 2005). However, the analytical methods used for biochemical and structural studies of kinase regulators present unique challenges. In most cases, they require the use of a complex matrix for their purification and further analysis (such as HPLC-MS) to determine their levels and sites of post-translational modifications (Kaiser *et al.,* 2001; Meijer et al. 2000). Therefore, the identification of kinase regulators and the choice of suitable analytical methods are essential for the study

of kinase-associated signal transduction.

Naturally occurring phenolic compounds, such as coumarins and their derivatives, have long been known for their medicinal properties (Bohm *et al.,* 1961; Cottiglia *et al.,* 2001; Fylaktakidou *et al.,* 2004; Murray *et al.,* 1982). In addition, their ability to inhibit or activate numerous enzymatic path- ways involved in disease rendered them very attractive for drug design (Bansal *et al.,* 2009; Riveiro *et al.,* 2008; Ryu *et al.,* 2010; Zhou *et al.,* 2008). In plants, coumarin derivatives are known to be key components of the defense response against abiotic and biotic stresses (Murray *et al.,* 1982; Zhou and Ibrahim 2010). As with most phenolic compounds, coumarins are derived from L-phenylalanine through the shikimate pathway by the action of phenylalanine deaminase/ ammonia-lyase (Koukol and Conn 1961) to form trans-cinnamic acid. Earlier reports of O-hyroxylation of the latter (Kosuge and Conn 1959) followed by its cyclization and substitution to coumarin derivatives have now to be revised. Recent findings indicate that coumarin derivatives are biosynthesized via O-hydroxylation of substituted cinnamoyl CoA esters by a 2-oxoglutarate-dependent dioxygenase.

The effects of coumarin and its derivatives, daphnetin (7,8- dihydroxycoumarin), esculetin (6,7-dihydroxycoumarin), 2-hydroxycoumarin, 4-hydroxycoumarin, and 7-hydroxycoumarin, on the activities of tyrosin specific protein kinase, epithelium growth factor (EGF) receptor, and serine- or threonine- specific protein kinases including cAMP-dependant protein kinase (PKA) and protein kinase C (PKC) were monitored in vitro. The results showed that daphnetin was the only effective protein kinase inhibitor among these coumpounds (Yang *et al.,* 1999). However inhibition of EGF-induced tyrosine phosphorylation by daphnetin was not observed in vivo with human hepatocellular carcinoma (Yang et al. 1999). On the other hand, we previously reported that: (i) LT treatment increases kinase

activity and stimulates protein phosphorylation in wheat (Vazquez-Tello et al. 1998), (ii) cold acclimation or high light stimulates the expression of a gene encoding a O-methyltransferase (TaOMT1) that methylates daphnetin to yield 8-methyl daphnetin (NDong *et al.*, 2003). Consequently, these data led us to consider daphnetin methylation by TaOMT1 as a means of modulating kinases during exposure to LT. Furthermore, we hypothesized that daphnetin methylation may alter the phosphorylation state or the activity of specific kinases, thus activating the expression of specific genes and (or) proteins or other metabolites involved in the CA response.

To identify the targeted kinases that interact with daphnetin or its derivatives, we used a suitable daphnetin-derivative as an affinity chromatography ligand to isolate and identify the interacting kinases. The results revealed that a phosphoribulokinase (EC 2.7.1.19) is the major daphnetin target in wheat. The corresponding gene encoding this enzyme was cloned, and its regulation during cold acclimation of wheat was determined. Furthermore, we tested the effect of daphnetin and its 7-methyl derivative on the activity of both endogenous and bacterially expressed phosphoribulokinase (PRK). Finally the presence of daphnetin and its methyl derivatives were monitored in wheat leaf extracts.

A.4 MATERIALS AND METHODS

A.4.1 Growth conditions

Seeds of winter wheat (*Triticum aestivum, L cv* Norstar) were germinated and grown in controlled environmental growth chambers (GCW15 chamber, Environmental Growth Chambers, Chargin Falls, Ohio, USA), with light intensity of 250 $\mu mol.m^{-2}\ s^{-1}$, 50 - 70% relative humidity, a 16-h photoperiod and day/night temperature regimes of 20/16 ^{O}C for non acclimated (NA) and 5/5 ^{O}C for cold acclimated (CA) plants. The seedlings were grown during 25 days for NA and 75 days for CA in coarse vermiculite in 500 mL-sized plastic pots at a density of three plants per pot and fertilized with Hoagland solution (Kröl, 1984).

A.4.2 Synthesis of 7,8-dihydroxycoumarin-4-acetic acid

Pyrogalol (2.51g, 20 mmol) was dissolved in sulfuric acid (10 mL) at 0 °C and 1,3-acetone dicarboxylic acid (3.21g, 22 mmol) was added in small portions. The mixture was allowed to warm up to room temperature and stirred for 3 h. The resulting yellowish suspension was poured onto crushed ice, and extracted (3x) with ethyl acetate. The combined organic layers were washed with brine, dried over $MgSO_4$ and the solvent was removed under vacuum. The crude product was titrated with ethyl acetate and filtered to afford 7,8-dihydroxycoumarin-4-acetic acid as a white powder (0.72 g, 15% yield), as colorless fine prisms (EtOH), mp 212.0-214.0 °C (dec.); lit. 204-205 °C, or 214-215 °C (dec.). FABMS (negative,TEA matrix) m/z (relative int. %) 235 $[M-H]^-$ (23), 191 $[M-H-CO_2]^-$ (73). ^{13}C NMR (67.5 MHz,

DMSO-d_6) 37.43, 111.95, 112.12, 112.24, 115.55, 132.28, 143.50, 149.48, 150.55, 160.15, 170.74. 39.50.

A.4.3 Daphnetin-derivative affinity chromatography preparation

EAH Sepharose-4B (GE healthcare, UK) with free amino groups at the end of its 10-atom spacer arm, was used to couple the ligand containing carboxyl groups with the carbodiimide EDC (N-ethyl-N'- (3-dimethylaminopropyl) carbodiimide hypochloride, 1.9 g/ml) diluted in distilled water, pH 4.5, was used to couple the daphnetin-derivative with EAH Sepharose 4B (Figure 1). The coupling reaction was stopped after 16 h and the resin was washed successively with 0.1 M sodium acetate, pH 4.5 and 0.1 M Tris-HCl, pH 8.3 containing 0.5 M NaCl. After centrifugation at 1500x*g* for 5 min, the supernatant was removed and the resin suspended in buffer A (20 mM of MOPS pH 7.2, 25 mM ß-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM CaCl2, 1 mM PMSF, 2.5 mM EDTA, 0.4 % (v/v) protease inhibitor cocktail (Calbiochem). The resin without daphnetin-derivative was used as a control.

A.4.4 Purification of phosphoribulokinase

A.4.4.1 Homogenization of plant tissues

Cold acclimated wheat leaves (ca. 40g) were homogenized with 150 mL of buffer B (20 mM of MOPS pH 7.2, 25 mM ß-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM $CaCl_2$, 1 mM PMSF, 10 mM EDTA, 0.4% (v/v) protease inhibitor cocktail (Calbiochem). After filtration on Miracloth, the homogenate was centrifuged

at 12,500x*g* for 30 min at 4 °C. The supernatant was saturated with 50% (w/v) ammonium sulphate, and the precipitated proteins were suspended in buffer A and dialyzed for 18h against the same buffer.

A.4.4.2 Diethyaminoethyl (DEAE) cellulose-column chromatography

The DEAE-cellulose (Bio-Rad) resin was equilibrated in buffer A before loading in a 15x2.5 cm column. Fraction from the dialysis after homogenization was loaded onto the DEAE-column, and washed with 1 volume of buffer A. The bound proteins were eluted with 0.5 M NaCl.

A.4.4.3 Daphnetin affinity chromatography

Proteins (0.5 mg) eluted from DEAE-column were loaded onto each of the daphnetin-derivative affinity and the control columns. Each column was washed with 150 µL buffer A. Since daphnetin had been showed to be a competitive inhibitor of tyrosine kinase protein (Yang *et al.*, 1999), proteins were eluted with 150 µL buffer A containing 10 mM ATP and 10 mM $MgCl_2$. A subsequent elution with KCl (0.5 M) was applied to the columns for more stringency, and the eluted fractions were dialyzed twice for 1 h against buffer A at 4 °C.

A.4.5 Identification of daphnetin interactors by mass spectrometry

Proteins eluted with ATP from the daphnetin-derivative and control affinity columns were separated by SDS-PAGE (Danyluk *et al.*, 1998) before being subjected to mass spectrometric analysis (Perkins *et al.*, 1999).

A.4.6 Production of wheat recombinant phosphoribulokinase

The wheat phosphoribulokinase (*TaPRK* >gi|21840|) open-reading frame was amplified using the primers ATCTCGAGTTATGGCATTCTGCAGCCCACACACC and ATTGAATTCTTTCAAACTTTTGCTGCTTCAGCAGG containing restriction site of XhoI in 5' and for EcoRI in 3'. The amplified cDNA was digested with restriction enzymes XhoI and EcorI, and subcloned in pTrcHis-C vector (Novagen) carrying ampicillin resistance and a polyhistidine tag. The constructs were electroporated in *DH5* cells of *Escherichia coli* contained in Super Optimal broth with Catabolite repression (SOC) medium. The ampicillin-resistant clones were transferred to Luria-Bertani (LB) medium containing 100 µg/mL of ampicillin. The bacterial cells were grown to an A_{600} of 0.5 at 37 °C and induced with isopropyl-ß-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, then harvested 3 h later (Ndong *et al.,* 2003). The cells were collected by centrifugation at 8,000x*g* for 10 min, and resuspended in 100 mM of Tris-HCl, pH 8.0. The mixture was sonicated using a sonic dismembrator 550 (Fisher Scientific) for 1min and 40 s then centrifuged at 10,000x*g* for 10 min at 4°C. The clear supernatant containing the recombinant PRK was then used for enzymes assays.

A.4.7 Phosphoribulokinase assay

The enzyme assays were carried out in 100 mM Tris-HCl pH 8.0 containing, 21% glycerol, 15.7 mM $NaHCO_3$, 0.16 µCi sodium bicarbonate ^{14}C, 1 mM MgCl2, 0.2 mM ATP, 21 mM DTT, 0.45 mM D-ribulose 5-phosphate (Ru5P) and 50 µg ribulose 1,5-biphosphate carboxylase (RUBISCO-Calbiochem) in a total volume of

210 µL. A volume of 50 µL of recombinant phosphoribulokinase was added to the mixture to initiate the enzyme reaction and then incubated for 12 min at 37 °C. The reaction was stopped by the addition of 200 µL of 2 M HCl. The untrapped bicarbonate was evaporated at 100 °C for 1 h, then suspended in 200 µL of H_2O. Liquid scintillation was carried out to determine the remaining ^{14}C label (Paulsen and Lane, 1966). For determining the endogenous wheat PRK activity, 0.15 g of leaves was extracted at 4 ^0C with 1,2 mL of 100 mM Tris-HCl, pH 8.0 containing 1% polivinylpolypyrrolidone and 0.1 % ß-mercaptoethanol (ß-met). After centrifugation at 4,000x*g* for 10 min, the supernatant was desalted in a PD10 column (GE Healthcare, UK) equilibrated with 10 mM Tris-HCl buffer, pH 8.0 containing 0.1% ß-met (Kamber, 1998). A volume of 10 µL of protein eluted from the PD10 column was used for enzyme assays. The enzyme assay reaction buffer was the same as that described for the recombinant PRK activity, except that no RUBISCO was added.

A.4.8 Northern blot analysis

Total RNA (10 µg) samples were separated on formaldehyde-agarose gels. After electrophoresis, RNA was transferred to Hybond nylon membrane (Amersham) and hybridized with *TaPRK* ^{32}P-labeled cDNA insert. The washing and blotting were performed as previously described (Ndong *et al.,* 2003).

A.4.9 Extraction of leaf phenolic compounds

Wheat leaves (*ca.* 10 g) were ground in dry ice before extraction with 85% aqueous methanol. The methanolic extract was concentrated under vacuum, and the

aqueous layer was defatted with a mixture of hexanes to remove lipids and chlorophyll pigments. The aqueous extract was hydrolyzed with 2 M HCl in order to release the phenolic aglycones from their parent glycosides, followed by 3×liquid-liquid extraction with ethyl acetate. The combined organic layers were concentrated under vacuum, and the resulting residue dissolved in methanol for analysis.

A.4.10 High performance liquid chromatography-mass spectrometry analyses

Two Agilent Technologies HPLC-MS instrument systems were used for the identification of dimethyldaphnetin and other phenolic compounds in the plant extracts. System 1 consisted of an Agilent 6410 Triple Quad LC/MS/MS coupled with a photodiode array detection-electrospray ionization source, which was used in multiple reaction-monitoring (MRM) modes. This approach of MS/MS permitted specialized analysis with high selectivity and sensitivity, and allowed improving quantification sensitivity. Samples were subjected to chromatography on an Agilent-XDB-C18 column (4.6 mm i.d. × 50 mm; particle size 1.8 μm), eluted with a linear gradient of 40% to 90% methanol in 0.1% formic acid and a flow rate of 0.35 ml.min^{-1} for 30 min. System 2 was composed of an Agilent 6210 Time-of-Flight LC/MS coupled with electrospray ionization- time-of flight analyzers ESI-TOF, which allowed a greater analytical confidence confirming the elemental composition and molecular formulae of intact compounds. The technical error of the time-of-flight mass spectrometer in terms of mass accuracy was 2 ppm. Samples (ca. 10 μL) were chromatographed on an Agilent SB-C18 column (2.1 mm id × 50 mm; particle size 3.5 μm), eluted with a linear gradient of 10% to 90% methanol in 0.1% formic acid at a flow rate of 0.4 ml.min^{1} for 20 min. For MS/MS and MRM analyses, several collision energies (CE) were tried in order to optimize the analysis method. For dimethyldaphnetin 25 eV was chosen as the optimum energy of collision in the

positive mode that gave rise to a parent ion peak at 207 and a maximum daughter ion peak at 163. The same energy of collision was used to analyze 8-hydroxy-7-methoxycoumarin, where 193 and 150 were chosen for the parent and daughter ion peaks, respectively. Reference standards were used to compare the corresponding retention times and mass profiles for each phenolic compound. Daphnetin, 8-hydroxy-7-methoxycoumarin and dimethyldaphnetin were purchased from Indofine Chemical Company (Hillsborough, NJ).

A.5 RESULTS

A.5.1 Purification and mass spectrometric identification of phosphoribulokinase

The total protein from cold acclimated wheat leaves was precipitated with ammonium sulphate and dialyzed before loading onto a DEAE-cellulose column for purification. Proteins bound to the column were eluted with 0.5 M NaCl (Figure S1). Equal amounts of the latter fraction were loaded onto both the EAH-sepharose coupled with the daphnetin-derivative ligand (Figure 1) and the control columns. Proteins eluted with ATP and those eluted with KCl from both columns, were digested with trypsin and further analyzed by mass spectrometry. ATP-eluted proteins from the daphnetin affinity column revealed the presence of several kinases, as well as other proteins specifically bound to the daphnetin ligand (Table 1), thus suggesting their potential interaction with daphnetin. Prediction by MASCOT (www.matrixscience.com), which compares the peptides identified by mass spectrometric analysis with the *Viridiplantae taxa*, indicated that among all the peptides attached to the daphnetin ligand, a putative conserved domain, of 21 amino acids was detected with a significant score (Table 1). A BLASTp search against the NCBI non-redundant database revealed that this peptide is 100% similar (E-value $1e^{-10}$) to a phosphoribulokinase sequence found in different plant species, including: *Triticum aestivum*, *Oryza sativa, Zea mays, Pisum sativum* and *Arabidopsis thaliana*. Two other peptides similar to protein kinases of the serine/threonine from *O. sativa* and *Brassica oleracea,* and one peptide of a receptor kinase-like from *O. sativa* were also found, but their scores of prediction were not significant. On the other hand, only RUBISCO was present in the KCl-eluted fraction from the daphnetin affinity column and the ATP-eluted fraction from the control resin (data not shown).

A.5.2 Effect of daphnetin and 7-methyldaphnetin on the activities of wheat and recombinant phosphoribulokinase

The TaPRK complete sequence (gi|21840|) was used to express the recombinant protein in bacteria. The expressed protein was confirmed by mass spectrometry (Perkins *et al.,* 1999) and enzyme assays were carried out in the presence of daphnetin and its 7-methyl derivative (Figure 2). The latter was used as an analog of 8-methyldaphnetin, that was not available commercially. The results show that daphnetin inhibits both the purified endogenous and bacterially expressed TaPRK proteins in a specific- and dose-dependant manner. The calculated daphnetin IC50 for the recombinant PRK (0.97 mM) is lower than that determined in the endogenous, partially purified wheat PRK (7.71 mM). This difference is due to the higher purity of the bacterially expressed protein as compared to the endogenous one. On the other hand, 7-methyldaphnetin did not inhibit either form of the phosphoribulokinases. This result indicates that daphnetin methylation abolishes the inhibitory effect of daphnetin and suggests its involvement in modulating the activity of wheat phosphoribulokinase.

A.5.3 Expression and activity of wheat phosphoribulokinase during cold acclimation

Since methylation of daphnetin occurs during cold acclimation and TaPRK bound to the daphnetin ligand on the affinity column, we investigated whether TaPRK (mRNA and/or enzyme activity) may be modulated by low temperature exposure. Northern blot analysis and activity measurement showed that both the TaPRK transcript level and enzyme activity were similar in both control and CA plants (Figure 3A & 3C). This indicates that LT has no effects on both the transcript and the activity level of the enzyme.

A.5.4 Identification of daphnetin and its methyl derivatives in wheat

HPLC-MS systems were used to identify daphnetin, its methyl derivatives and other phenolics in wheat plant extracts. Although daphnetin itself was not detected, however, its dimethyl derivative, 7,8-dimethoxycoumarin, was observed in both the control and cold acclimated plants (Figure 4B). Using the MRM technique demonstrates that the dimethylated form of daphnetin accumulates to at least 20- fold more than the monomethyl derivative (Figures 4A & 5). The comparison of their spectral data with available reference compounds, allowed their identification with a high degree of certainty. To our knowledge, this is the first report of the isolation and identification of 7,8-dimethoxycoumarin as a natural constituent of wheat leaf extracts.

A.6 DISCUSSION

Using the daphnetin ligand as an affinity column allowed the isolation and identification of a phosphoribulokinase, a protein that may interact with daphnetin or its derivatives in wheat during CA. Phosphoribulokinase has the ability to interact with several molecules. In Arabidopsis, the interaction of phosphoribulokinase with glyceraldehyde phosphate dehydrogenase (GAPDH) during light–dark conditions was proposed as a means of regulation of the Calvin cycle (Graciet *et al.,* 2004; Marri *et al.,* 2005). The activities of both PRK and GAPDH decreased considerably when associated with the regulatory peptide CP12, to form a supramolecular complex. The dissociation of these proteins with reduced thioredoxin, NADP, 1,3-iso-phosphoglycerate (BPGA) or ATP resulted in the activation of PRK (Marri *et al.* 2005). Although PRKs in higher plants are regulated by a thioredoxin mediated thiol/disulfide exchange, it was suggested that the formation of complexes involving kinases could be a potential factor of regulation under stress conditions (Marri *et al.,* 2005). Because plant kinases form a large protein family, the identification of a specific kinase associated with cold acclimated plants was quite a challenge, especially since TaPRK belongs to the nucleoside monophosphate kinase family of enzymes (Runquist and Miziorko 2006). Our enzyme activity data show that the endogenous or bacterially expressed TaPRK activity was inhibited in a specific- and dose-dependent manner by daphnetin, but not by either of its methylated derivatives. Furthermore, TaPRK enzyme activity measurements showed no significant difference between NA and CA wheat leaves. This suggests that cold acclimation did not alter the activity of this key enzyme but maintained its level to that of the control plants. PRK is an important light-regulated enzyme of the Calvin cycle that is involved in ensuring

a sufficient supply of RuBP for the continuous fixation of CO_2 in light by RUBISCO. Stud- ies of different plant species have shown that photosynthetic genes are down-regulated during cold stress, and consequently photosynthesis is inhibited by either low temperature or high light (Ndong *et al.,* 2001; Wang *et al.,* 2009). It is well established that both LT stress and high light can induce photoinhibition that reduces the rates of photosynthetic carbon assimilation. In contrast to LT stress, cold- acclimated cereals, which have grown and developed at low temperature, exhibit an enhanced photosynthetic capacity and a resistance to photoinhibition (Öquist and Hüner 2003). The alleviation of phopshoribulokinase inhibition by methylated daphnetin is significant for the Calvin cycle turnover, since more ribulose 1,5-biphosphate can be formed. We suggest, therefore, that the modulation of daphnetin methylation may play an important role in regulating CO_2 assimilation in cold-acclimated cereals through its modulation of phosphoribulokinase activity by preventing any inhibition of its activity during growth at LT.

A.7 ACKNOWLEDGMENTS

This work was supported by funds from the Natural Sciences and Engineering Research Council of Canada (NSERC) discovery grants to (FS), (NH) & (RKI) and a Canada Research Chair (CRC) in Therapeutic Chemistry (RR).

Table A.1: Proteins identified by mass spectrometric analysis from the EAH sepharose-daphnetin ligand after elution with ATP.

Accession number	Name	Peptide	Species	Score
gi|21839	Phosphorubilokinase	**HADFPGSNNGTGLFQTIVGLK**	*Triticum aestivum*	50
gi|1885326	Phosphorubilokinase	**HADFPGSNNGTGLFQTIVGLK**	*Pisum sativum*	50
gi|23197622	Phosphorubilokinase	**HADFPGSNNGTGLFQTIVGLK**	*Arabidopsis thaliana*	50
gi|38347079	Phosphorubilokinase	**HADFPGSNNGTGLFQTIVGLK**	*Oryza sativa*	50
gi|50947343	Putative wall-associated serine/threonine kinase	TTNILLDDRFIAK **ADNAMLLQQEVLSR**	*Oryza sativa*	30
gi|16304001	Auxin efflux carrier protein	QAAIGMLIR	*Narcissus pseudonarcissus*	28
gi|37532440	Putative retroelement	MGERGQPFFALLK FISSDELPEDDAEAE	*Oryza sativa*	28
gi|3415113	Villin 1	YTYKER SEFYALPKWK	*Arabidopsis thaliana*	28
gi|38260683	Pollen coat oleosin-glycine rich protein	MAPFPLSLILGK	*Arabidopsis arenosa*	27
gi|50915964	Receptor protein kinase-like	VGDFGFAR KPPLPGPGSDQGK	*Oryza sativa*	26
gi|21655205	Putative TIR/NBS/LRR	VLGGLLRGK	*Pinus taeda*	25
gi|34897326	Putative RNA helicase	**GGRGGWDSDGEDR**	*Oryza sativa*	25
gi|5817734	Maturase-like protein	**SRHQDFLYPLIFR** **SQMLQNSFLMEIVMKK**	*Abrus precatorius*	25
gi|34495199	Arginine decarboxylase	VIDIGGGLGIDYDGTK	*Lotus corniculatus*	25
gi|2181190	Serine/threonine kinase	**MLVYEYMPNK** **GGFGPVYKGVLEDGQEIAVK**	*Brassica oleracea*	25

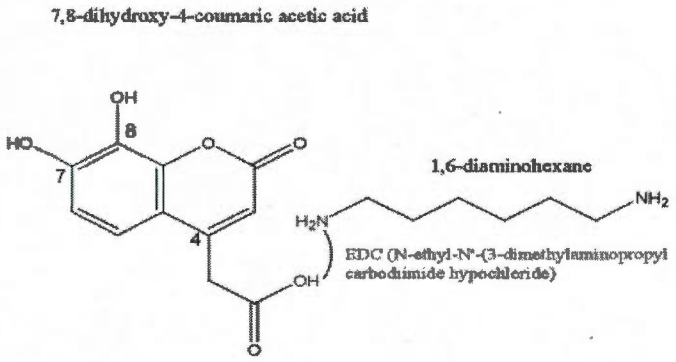

Figure A.1: Coupling of daphnetin-derivative to EAH sepharose column. Daphnetin-derivative (7,8-dihydroxy-4-coumarin) acetic acid was synthesized and coupled with an EAH Sepharose using EDC as described in Methods section. Proteins eluted with ATP were dialyzed and subjected to mass spectrometric analysis.

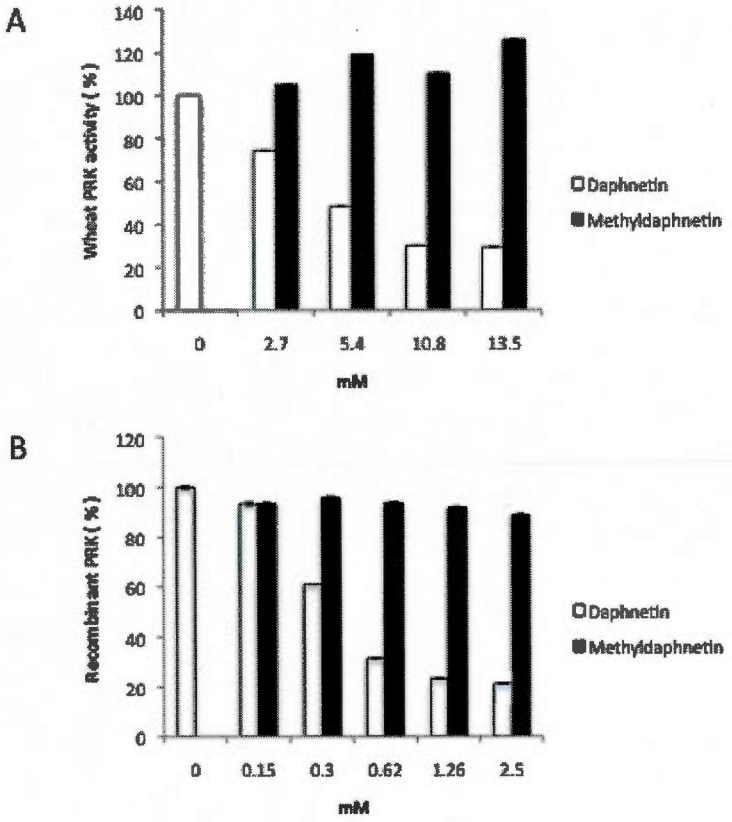

Figure A.2: Enzyme activity assays of endogenous and recombinant wheat phosphoribulokinase (PRK) in the presence of daphnetin and 7-methyldaphnetin. A. Assays using partially purified phosphoribulokinase from wheat. Activity is represented by percentage where 100% equals 4,74 nKat/ml of PRK extract. Error bars represent the standard deviation of two biological replicates. B. Assays using recombinant phosphoribulokinase. Activity is represented as percentage, where 100% is equivalent of 6,18 nKat/ml of PRK extract. Error bars represent the standard deviation of two different extractions.

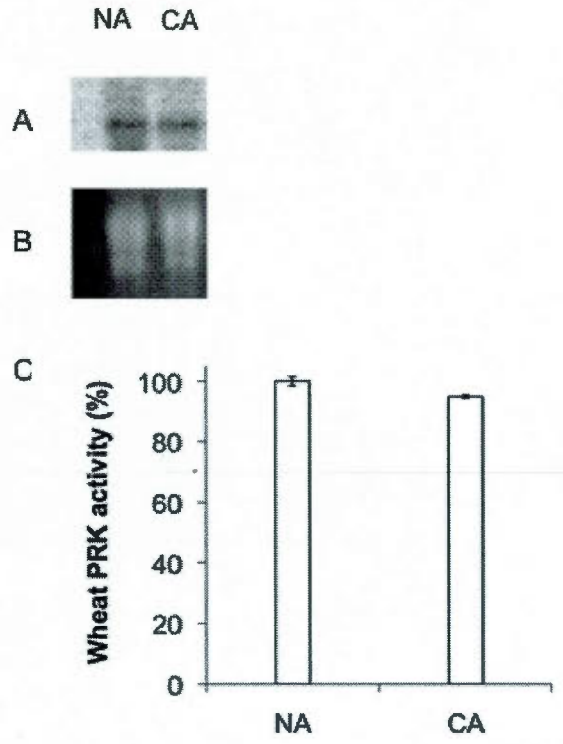

Figure A.3A: Northern blot analysis of wheat phosphoribulokinase in NA (25 days) and CA (75 days) plants. 3B. 18S ribosomal RNA gel. C. Enzyme Activity assays of the wheat phosphoribulokinase during LT- exposure. Wheat phosphoribulokinase activity was monitored in NA (25 days) and CA (75 days) plants. Activity is represented by percentage where 100% equals 26.7 nkat/g fresh weight. NA: Non Acclimated; CA: Cold Acclimated.

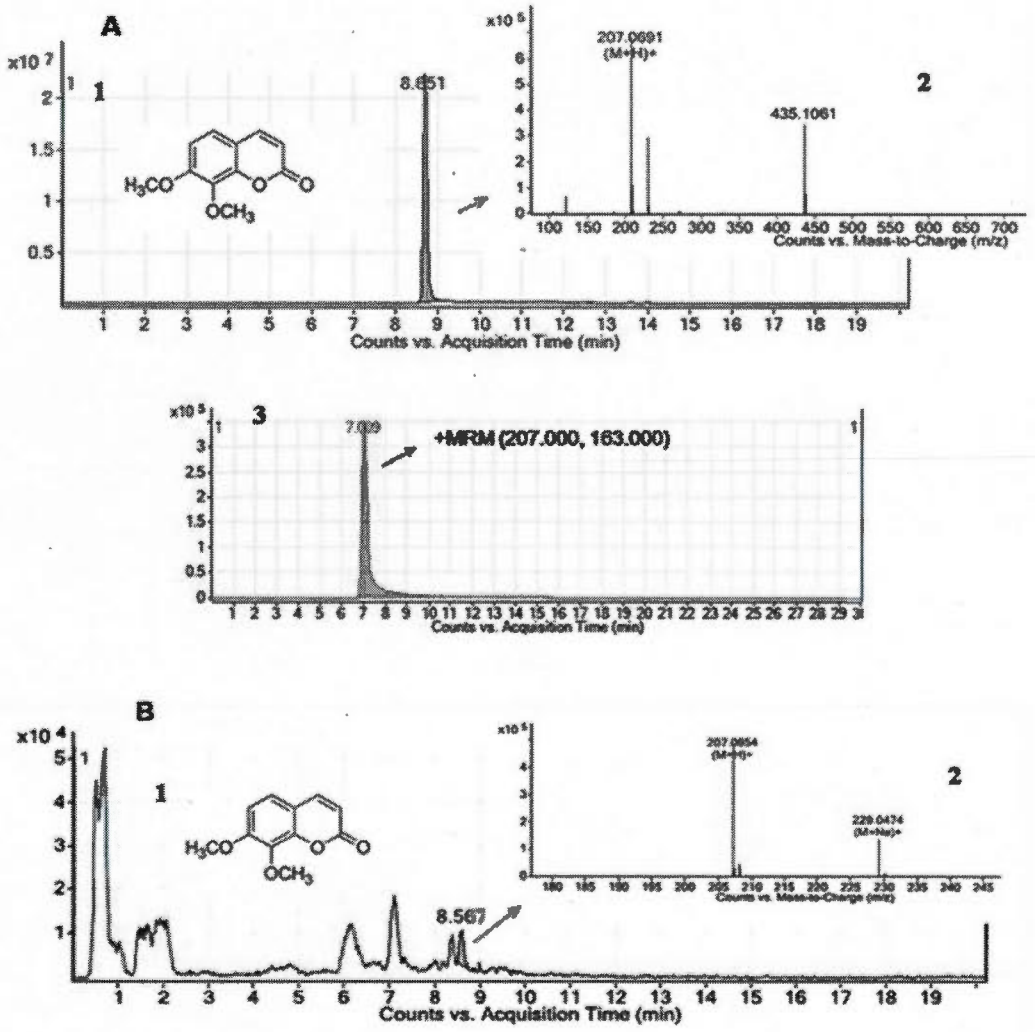

Figure A.4: Identification of dimethyldaphnetin in winter wheat leaf extracts. ESI-MS data of both dimethyldaphnetin standard (A) and dimethyldaphnetin from wheat extract (B): (1) Retention time appearing at 8.6 min, (2): molecular ion [M+H]+ appearing at m/z 207.05 , (3) : +MRM of products ions 207->163. For (1) and (2), an Agilent SB-C18 column (2.1 id ×30 mm; particle size 3.5µm) was used coupled with ESI-MS mass-spectrometric detector time of flight analysers ESI-TOF (System 2). For MRM results (3) LC-MS Agilent–XDB-C18 column (4.6 id x 50 mm) was used coupled with photodiode array detector - electrospray ionization and quadrupole (System 1).

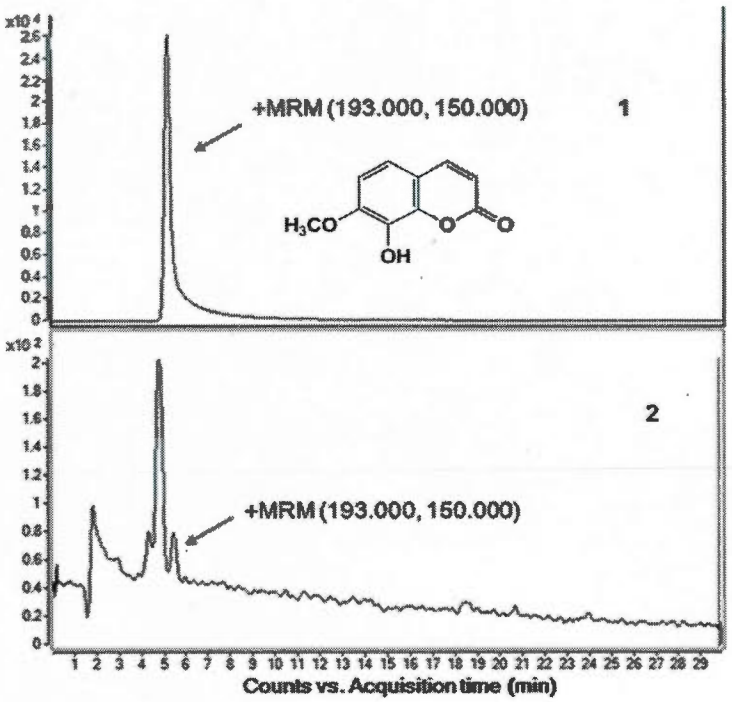

Figure A.5: Identification of monomethyldaphnetin in winter wheat leaf extracts. MRM of products ions 193-> 150 for reference (1) and in wheat extract (2) at Rt 5.1 min.

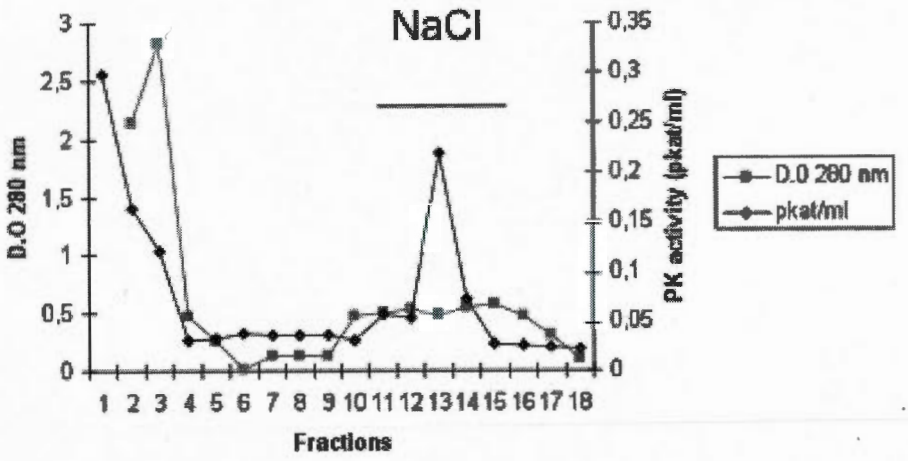

Figure A.S1: Purification of wheat protein kinase on DEAE-cellulose chromatography. Proteins were extracted from cold- acclimated (CA) wheat. After precipitation with ammonium sulphate (50% saturation), protein kinases were chromatographed on a DEAE-cellulose column. The bound proteins were eluted with 0.5 M NaCl. The activity of protein kinases is represented in pkat/ml. Protein content was measured by absorbance at 280 nm.

A.8 REFERENCES

Allard, F., Houde, M., Kröl, M., Ivanov, A., Hüner, N., and Sarhan, F. 1998. Betaine improves freezing tolerance in wheat. Plant Cell physiol. **39**(11): 1194.

Bansal, Y., Ratra, S., Bansal, G., Singh, L. and Aboul-Enein, H.Y. 2009. Design and synthesis of coumarin substituted oxathiadiazolone derivatives anti-inflammatory activity possibly through p38 MAP Kinase inhibition J. Iran. Chem. Soc **6**(3).

Bohm, B.A., Ibrahim, R.K. and Towers G.H.N. 1961. The partial elucidation of the structure of a new coumarin from *Hydrangea macrophylla ser*. Biochem. Cell Biol. **39**.

Chevalier, D., Batoux, M., Fulton, L., Pfister, K., Yadav, R.K., Schellenberg, M., and Schneitz, K. 2005. Strubbelig defines a receptor kinase-mediated signaling pathway regulating organ development in Arabidopsis. Proc. Natl. Acad. Sci. U S A **102**(25): 9074-9079.

Colcombet, J., Boisson-Dernier, A., Ros-Palau, R., Vera, C.E., and Schroeder, J.I. 2005. Arabidopsis somatic embryogenesis receptor kinases 1 and 2 are essential for tapetum development and microspore maturation. Plant Cell **17**(12): 3350-3361.

Cottiglia, F., Loy, G., Garau, D., Floris, C., Casu, M., Pompei, R., and Bonsignore, L. 2001. Antimicrobial evaluation of coumarins and flavonoids from the stems of *Daphne gnidium L.* Phytomedicine **8**(4): 302-305.

Danyluk, J., Perron, A., Houde, M., Limin, A., Fowler, B., Benhamou, N., and Sarhan, F. 1998. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell **10**(4): 623.

Fylaktakidou, K.C., Hadjipavlou-Litina, D.J., Litinas, K.E., and Nicolaides, D.N. 2004. Natural and synthetic coumarin derivatives with anti-inflammatory/ antioxidant activities. Curr. Pharm. Des. **10**(30): 3813-3833.

Graciet, E., Lebreton, S., and Gontero, B. 2004. Emergence of new regulatory mechanisms in the Benson-Calvin pathway via protein-protein interactions: a glyceraldehyde-3-phosphate dehydrogenase/CP12/phosphoribulokinase complex. J. Exp. Bot. **55**(400): 1245-1254.

Houde, M., Danyluk, J., Laliberte, J.F., Rassart, E., Dhindsa, R.S., and Sarhan, F. 1992. Cloning, characterization, and expression of a cDNA encoding a 50-

kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol. **99**(4): 1381-1387.

Kaiser, A., Nishi, K., Gorin, F.A., Walsh, D.A., Bradbury, E.M., and Schnier, J.B. 2001. The cyclin-dependent kinase (CDK) inhibitor flavopiridol inhibits glycogen phosphorylase. Arch. Biochem. Biophys. **386**(2): 179-187.

Kamber, L., and Feller, U. 1998. Influence of the activation status and of ATP on phosphoribulokinase degradation. J. Exp. Bot. **49**(310): 139-144.

Kröl, M.G., M. Hüner, NPA. . 1984. An appropriate physiological control for environmental temperature studies: comparative growth kinetics of winter rye. Can. J. Bot. **62**: 1062-1068.

Marri, L., Trost, P., Pupillo, P., and Sparla, F. 2005. Reconstitution and properties of the recombinant glyceraldehyde-3-phosphate dehydrogenase/CP12/phosphoribulokinase supramolecular complex of Arabidopsis. Plant Physiol. **139**(3): 1433-1443.

Meijer, L., Thunnissen, A.M., White, A.W., Garnier, M., Nikolic, M., Tsai, L.H., Walter, J., Cleverley, K.E., Salinas, P.C., Wu, Y.Z., Biernat, J., Mandelkow, E.M., Kim, S.H., and Pettit, G.R. 2000. Inhibition of cyclin-dependent kinases, GSK-3beta and CK1 by hymenialdisine, a marine sponge constituent. Chem. Biol. **7**(1): 51-63.

Murray, R., Mendez,D.H., et Brown, S.A. 1982. The natural coumarins: occurence, chemistry and biochemistry. Wiley & Sons, New York.

Ndong, C., Anzellotti, D., Ibrahim, R.K., Hüner, N.P., and Sarhan, F. 2003. Daphnetin methylation by a novel O-methyltransferase is associated with cold acclimation and photosystem II excitation pressure in rye. J. Biol. Chem. **278**(9): 6854-6861.

Ndong, C., Danyluk, J., Hüner, N.P.A, and Sarhan, F. 2001. Survey of gene expression in winter rye during changes in growth temperature, irradiance or excitation pressure. Plant Mol. Biol. **45**(6): 691-703.

Öquist, G., and Hüner, N. 2003. Photosynthesis of overwintering evergreen plants. Annu. Rev. Plant Biol. **54**: 329-355.

Paulsen, J.M., and Lane, M.D. 1966. Spinach ribulose diphosphate carboxylase. I. Purification and properties of the enzyme. Biochemistry **5**(7): 2350-2357.

Perkins, D.N., Pappin, D.J., Creasy, D.M., and Cottrell, J.S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20**(18): 3551-3567.

Poulton, J.E., McRee, D.E., and Conn, E.E. 1980. Intracellular localization of two enzymes involved in coumarin biosynthesis in *Melilotus alba*. Plant Physiol. **65**(2): 171.

Riveiro, M.E., Vazquez, R., Moglioni, A., Gomez, N., Baldi, A., Davio, C., and Shayo, C. 2008. Biochemical mechanisms underlying the pro-apoptotic activity of 7,8-dihydroxy-4-methylcoumarin in human leukemic cells. Biochem. Pharmacol. **75**(3): 725-736.

Runquist, J.A., and Miziorko, H.M. 2006. Functional contribution of a conserved, mobile loop histidine of phosphoribulokinase. Protein Sci. **15**(4): 837-842.

Ryu, Y.B., Kim, J.H., Park, S.J., Chang, J.S., Rho, M.C., Bae, K.H., Park, K.H., and Lee, W.S. 2010. Inhibition of neuraminidase activity by polyphenol compounds isolated from the roots of *Glycyrrhiza uralensis*. Bioorg. Med. Chem. Lett. **20**(3): 971-974.

Stafford, H.A. 1961. Compartmentation in natural product biosynthesis by multienzyme complexes. in; The Biochemistry of Plants. E.E. Conn (Ed.), vol. 7, pp. 118-138. Academic Press, New York.

Stone, J.M., and Walker, J.C. 1995. Plant protein kinase families and signal transduction. Plant Physiol. **108**(2): 451-457.

Vazquez-Tello, A., Ouellet, F., and Sarhan, F. 1998. Low temperature-stimulated phosphorylation regulates the binding of nuclear factors to the promoter of Wcs120, a cold-specific gene in wheat. Mol. Gen. Genet. **257**(2): 157-166.

Vlad, F., Turk, B.E., Peynot, P., Leung, J., and Merlot, S. 2008. A versatile strategy to define the phosphorylation preferences of plant protein kinases and screen for putative substrates. Plant J. **55**(1): 104-117.

Wang, X., Goshe, M.B., Soderblom, E.J., Phinney, B.S., Kuchar, J.A., Li, J., Asami, T., Yoshida, S., Huber, S.C., and Clouse, S.D. 2005. Identification and functional analysis of in vivo phosphorylation sites of the Arabidopsis brassinosteroid-insensitive1 receptor kinase. Plant Cell **17**(6): 1685.

Wang, X., Pingfang, Y., Zhang, X., Xu, Y., Kuang, T., Shen, S. and He, Y. 2009. Proteomic analysis of the cold stress response in the moss, *Physcomitrella patens*. Proteomics **9**: 4529-4538.

Winfield, M.O., Lu, C., Wilson, I.D., Coghill, J.A., and Edwards, K.J. 2010. Plant responses to cold: transcriptome analysis of wheat. Plant biotechnol. J. **8**(7): 749-771.

Winkel-Shirley, B. 2002. Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. **5**(3): 218-223.

Yang, E.B., Zhao, Y.N., Zhang, K., and Mack, P. 1999. Daphnetin, one of coumarin derivatives, is a protein kinase inhibitor. Biochem. Biophys. Res. Commun. **260**(3): 682-685.

Zhou, J.M., Gold, N.D., Martin, V.J.J., Wollenweber, E., and Ibrahim, R.K. 2006. Sequential O-methylation of tricetin by a single gene product in wheat. Biochim. Biophys. Acta **1760**(7): 1115-1124.

Zhou, J.M., and Ibrahim, R.K. 2010. Tricin-a potential multifunctional nutraceutical. Phytochemistry Reviews **9**(3): 413-424.

Zhou, X., Wang, X.B., Wang, T., and Kong, L.Y. 2008. Design, synthesis, and acetylcholinesterase inhibitory activity of novel coumarin analogues. Bioorg. Med. Chem. **16**(17): 8011-8021.

RÉFÉRENCES

1. Aalto, T., Vesala, T., Mattila, T., Simbierowicz, P. et Hari, P. 1999. A three-dimensional stomatal CO2 exchange model including gaseous phase and leaf mesophyll separated by irregular interface. J Theor Biol **196**: 115-128.

2. Adams III, W., Demmig-Adams, B., Rosenstiel, T., Brightwell, A. et Ebbert, V. 2002. Photosynthesis and photoprotection in overwintering plants. Plant Biol. **4**: 545-557.

3. Adamska, I. (2004). The Elip Family of Stress Proteins in the Thylakoid Membranes of Pro- and Eukaryota. In *Regulation of Photosynthesis* (Aro, E.-M. et Andersson, B., eds.), Vol. 11, pp. 487-505. Springer Netherlands.

4. Adamska, I., Ohad, I. et Kloppstech, K. 1992. Synthesis of the early light-inducible protein is controlled by blue light and related to light stress. Proc. Natl. Acad. Sci. USA **89**: 2610-2613.

5. Ahsan, N., Lee, D.G., Kim, K.H., Alam, I., Lee, S.H., Lee, K.W., Lee, H. et Lee, B.H. 2010. Analysis of arsenic stress-induced differentially expressed proteins in rice leaves by two-dimensional gel electrophoresis coupled with mass spectrometry. Chemosphere **78**: 224-231.

6. Ainsworth, E.A., Davey, P.A., Bernacchi, C.J., Dermody, O.C., Heaton, E.A., Moore, D.J., Morgan, P.B., Naidu, S.L., Yoo Ra, H. et Zhu, X. 2002. A meta analysis of elevated [CO2] effects on soybean (Glycine max) physiology, growth and yield. Global Change Biol. **8**: 695-709.

7. Ainsworth, E.A., Leakey, A.D.B., Ort, D.R. et Long, S.P. 2008. FACE-ing the facts: inconsistencies and interdependence among field, chamber and modeling studies of elevated [CO2] impacts on crop yield and food supply. New Phytol. **179**: 5-9.

8. Ainsworth, E.A. et Long, S.P. 2005. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of

photosynthesis, canopy properties and plant production to rising CO_2. New Phytol. **165**: 351-371.

9. Ainsworth, E.A. et Rogers, A. 2007. The response of photosynthesis and stomatal conductance to rising [CO2]: mechanisms and environmental interactions. Plant, Cell Environ. **30**: 258-270.

10. Ainsworth, E.A., Rogers, A., Vodkin, L.O., Walter, A. et Schurr, U. 2006. The effects of elevated CO_2 concentration on soybean gene expression. An analysis of growing and mature leaves. Plant Physiol. **142**: 135-147.

11. Albert, M., K Jehle, A., Lipschis, M., Mueller, K., Zeng, Y. et Felix, G. 2010. Regulation of cell behaviour by plant receptor kinases: pattern recognition receptors as prototypical models. Eur. J. Cell Biol. **89**: 200-207.

12. Allagulova, C.R., Gimalov, F., Shakirova, F. et Vakhitov, V. 2003. The plant dehydrins: structure and putative functions. Biochemistry **68**: 945-951.

13. Alonso, A., Queiroz, C.S. et Magalh√£es, A.C. 1997. Chilling stress leads to increased cell membrane rigidity in roots of coffee (*Coffea arabica* L.) seedlings. Biochimica et Biophysica Acta (BBA)-Biomembranes **1323**: 75-84.

14. Amasino, R. 2004. Vernalization, competence, and the epigenetic memory of winter. Plant Cell **16**: 2553-2559.

15. Amthor, J.S. 2000. Direct effect of elevated CO2 on nocturnal in situ leaf respiration in nine temperate deciduous tree species is small. Tree physiology **20**: 139.

16. Amthor, J.S. 2000. Direct effect of elevated CO2 on nocturnal in situ leaf respiration in nine temperate deciduous tree species is small. Tree physiology **20**: 139-144.

17. Andersson, A., Keskitalo, J., Sjodin, A., Bhalerao, R., Sterky, F., Wissel, K., Tandre, K., Aspeborg, H., Moyle, R., Ohmiya, Y., Brunner, A., Gustafsson, P., Karlsson, J., Lundeberg, J., Nilsson, O., Sandberg, G., Strauss, S., Sundberg, B., Uhlen, M., Jansson, S. et Nilsson, P. 2004. A transcriptional timetable of autumn senescence. Genome biology **5**: R24.

18. Antikainen, M., Griffith, M., Zhang, J., Hon, W.C., Yang, D.S.C. et Pihakaski-Maunsbach, K. 1996. Immunolocalization of antifreeze proteins in winter rye leaves, crowns, and roots by tissue printing. Plant Physiol. **110**: 845-857.

19. Armstrong, A.F., Logan, D.C., Tobin, A.K., O'Toole, P. et Atkin, O.K. 2006. Heterogeneity of plant mitochondrial responses underpinning respiratory acclimation to the cold in Arabidopsis thaliana leaves. Plant, Cell Environ. **29**: 940-949.

20. Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24**: 1-15

21. Asada, K. 1999. The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu. Rev. Plant Biol. **50**: 601-639.

22. Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. et Sheen, J. 2002. MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature **415**: 977-983.

23. Assmann, S.M. 1993. Signal transduction in guard cells. Annual Review of Cell Biology **9**: 345-375.

24. Atkin, O.K. et Tjoelker, M.G. 2003. Thermal acclimation and the dynamic response of plant respiration to temperature. Trends Plant Sci. **8**: 343-351.

25. Badawi, M., Danyluk, J., Boucho, B., Houde, M. et Sarhan, F. 2007. The CBF gene family in hexaploid wheat and its relationship to the phylogenetic complexity of cereal CBF s. Mol. Genet. Genomics **277**: 533-554.

26. Badawi, M., Reddy, Y.V., Agharbaoui, Z., Tominaga, Y., Danyluk, J., Sarhan, F. et Houde, M. 2008. Structure and functional analysis of wheat ICE (inducer of CBF expression) genes. Plant Cell Physiol. **49**: 1237.

27. Baker, J., Allen Jr, L., Boote, K. et Pickering, N.B. 2000. Direct effects of atmospheric carbon dioxide concentration on whole canopy dark respiration of rice.).

28. Baker, J., Boote, K. et Pickering, N.B. 2001. Direct effects of atmospheric carbon dioxide concentration on whole canopy dark respiration of rice. Global Change Biol. **6**: 275-286.

29. Baker, N.R. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu Rev Plant Biol **59**: 89-113.

30. Bäurle, I. et Dean, C. 2006. The timing of developmental transitions in plants. Cell **125**: 655-664.

31. Benedict, C., Skinner, J.S., Meng, R., Chang, Y., Bhalerao, R., Huner, N.P., Finn, C.E., Chen, T.H. et Hurry, V. 2006. The CBF1-dependent low temperature signalling pathway, regulon and increase in freeze tolerance are conserved in *Populus spp*. Plant Cell Environ **29**: 1259-1272.

32. Benko-Iseppon, A.M., Galdino, S.L., Calsa, T., Jr., Kido, E.A., Tossi, A., Belarmino, L.C. et Crovella, S. 2010. Overview on plant antimicrobial peptides. Curr Protein Pept Sci **11**: 181-188.

33. Bernacchi, C., Calfapietra, C., Davey, P., Wittig, V., Scarascia Mugnozza, G., Raines, C. et Long, S. 2003. Photosynthesis and stomatal conductance responses of poplars to free air CO2 enrichment (PopFACE) during the first growth cycle and immediately following coppice. New Phytol. **159**: 609-621.

34. Bernacchi, C.J., Kimball, B.A., Quarles, D.R., Long, S.P. et Ort, D.R. 2007. Decreases in stomatal conductance of soybean under open-air elevation of [CO2] are closely coupled with decreases in ecosystem evapotranspiration. Plant Physiol **143**: 134-144.

35. Bernacchi, C.J., Leakey, A.D., Heady, L.E., Morgan, P.B., Dohleman, F.G., McGrath, J.M., Gillespie, K.M., Wittig, V.E., Rogers, A., Long, S.P. et Ort, D.R. 2006. Hourly and seasonal variation in photosynthesis and stomatal conductance of soybean grown at future CO_2 and ozone concentrations for 3 years under fully open-air field conditions. Plant Cell Environ **29**: 2077-2090.

36. Bernier, G. et Périlleux, C. 2005. A physiological overview of the genetics of flowering time control. Plant Biotechnol. J. **3**: 3-16.

37. Berri, S., Abbruscato, P., Faivre-Rampant, O., Brasileiro, A., Fumasoni, I., Satoh, K., Kikuchi, S., Mizzi, L., Morandini, P. et PË, M.E. 2009. Characterization of WRKY co-regulatory networks in rice and Arabidopsis. BMC Plant Biol. **9**: 120.

38. Bertrand, A., Prevost, D., Bigras, F.J. et Castonguay, Y. 2007. Elevated atmospheric CO_2 and strain of rhizobium alter freezing tolerance and cold-induced molecular changes in alfalfa (*Medicago sativa*). Ann. Bot. **99**: 275-284.

39. Bidart-Bouzat, M.G., Mithen, R. et Berenbaum, M.R. 2005. Elevated CO_2 influences herbivory-induced defense responses of *Arabidopsis thaliana*. Oecologia **145**: 415-424.

40. Bigras, F. et Bertrand, A. 2006. Responses of *Picea mariana* to elevated CO_2 concentration during growth, cold hardening and dehardening: phenology, cold tolerance, photosynthesis and growth. Tree physiology **26**: 875-888.

41. Bischof, M., Eichmann, R. et Hückelhoven, R. 2011. Pathogenesis-associated transcriptional patterns in Triticeae. J. Plant Physiol. **168**: 1618-1328.

42. Bishop, J., Dean, A. et Mitchell-Olds, T. 2000. Rapid evolution in plant chitinases: molecular targets of selection in plant-pathogen coevolution. Proc. Natl. Acad. Sci. USA **97**: 5322-5327.

43. Blázquez, M.A., Ahn, J.H. et Weigel, D. 2003. A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. Nat. Genet. **33**: 168-171.

44. Bloom, A.J., Burger, M., Asensio, J.S.R. et Cousins, A.B. 2010. Carbon Dioxide Enrichment Inhibits Nitrate Assimilation in Wheat and Arabidopsis. Science **328**: 899-903.

45. Bloom, A.J., Smart, D.R., Nguyen, D.T. et Searles, P.S. 2002. Nitrogen assimilation and growth of wheat under elevated carbon dioxide. Proc. Natl. Acad. Sci. USA **99**: 1730-1735.

46. Boese, S.R. et Huner, N.P. 1992. Developmental history affects the susceptibility of spinach leaves to in vivo low temperature photoinhibition. Plant Physiol **99**: 1141-1145.

47. Boese, S.R. et Hüner, N.P. 1990. Effect of growth temperature and temperature shifts on spinach leaf morphology and photosynthesis. Plant Physiol **94**: 1830-1836.

48. Borovskii, G., Stupnikova, I., Antipina, A., Anuchina, O. et Voinikov, V. 2005. Association of dehydrins with wheat mitochondria during low-temperature adaptation. Russian Journal of Plant Physiology **52**: 194-198.

49. Brearley, J., Venis, M.A. et Blatt, M.R. 1997. The effect of elevated CO 2 concentrations on K+ and anion channels of *Vicia faba L.* guard cells. Planta **203**: 145-154.

50. Bryant, J., Taylor, G. et Frehner, M. 1998. Photosynthetic acclimation to elevated CO2 is modified by source: sink balance in three component species of chalk grassland swards grown in a free air carbon dioxide enrichment (FACE) experiment. Plant, Cell Environ. **21**: 159-168.

51. Buchanan-Wollaston, V. et Ainsworth, C. 1997. Leaf senescence in *Brassica napus*: cloning of senescence related genes by subtractive hybridisation. Plant Mol. Biol. **33**: 821-834.

52. Bunce, J.A. 2001. Effects of prolonged darkness on the sensitivity of leaf respiration to carbon dioxide concentration in C3 and C4 species. Ann. Bot. **87**: 463-468.

53. Casson, S.A., Franklin, K.A., Gray, J.E., Grierson, C.S., Whitelam, G.C. et Hetherington, A.M. 2009. phytochrome B and PIF4 regulate stomatal development in response to light quantity. Curr Biol **19**: 229-234.

54. Casteel, C.L., O'Neill, B.F., Zavala, J.A., Bilgin, D.D., Berenbaum, M.A.Y.R. et DeLucia, E. 2008. Transcriptional profiling reveals elevated CO2 and elevated O3 alter resistance of soybean (*Glycine max*) to Japanese beetles (*Popillia japonica)*. Plant, Cell Environ. **31**: 419-434 %@ 1365-3040.

55. Castonguay, Y., Nadeau, P., Lechasseur, P. et Chouinard, L. 1995. Differential accumulation of carbohydrates in alfalfa cultivars on contrasting winter hardiness. Crop Sci. **35**: 509-516.

56. Chen, G.-Y., Yong, Z.-H., Liao, Y., Zhang, D.-Y., Chen, Y., Zhang, H.-B., Chen, J., Zhu, J.-G. et Xu, D.-Q. 2005. Photosynthetic acclimation in rice leaves to free-air CO_2 enrichment related to both ribulose-1,5-bisphosphate carboxylation limitation and ribulose-1,5-bisphosphate regeneration limitation. Plant Cell Physiol. **46**: 1036-1045.

57. Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G. et Whitham, S.A. 2002. Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell **14**: 559-574.

58. Chinnusamy, V., Zhu, J. et Zhu, J.K. 2007. Cold stress regulation of gene expression in plants. Trends Plant Sci. **12**: 444-451.

59. Cleland, W.W., Andrews, T.J., Gutteridge, S., Hartman, F.C. et Lorimer, G.H. 1998. Mechanism of RUBISCO: the carbamate as general base. Chem. Rev. **98**: 549-562.

60. Cook, D., Fowler, S., Fiehn, O. et Thomashow, M.F. 2004. A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proc. Natl. Acad. Sci. USA **101**: 15243-15248.

61. Coplen, T.B. 1994. Reporting of stable hydrogen, carbon, and oxygen isotopic abundances. Pure Appl. Chem. **66**: 273-273.

62. Cousins, A. et Bloom, A. 2003. Influence of elevated CO2 and nitrogen nutrition on photosynthesis and nitrate photo assimilation in maize (Zea mays L.). Plant, Cell Environ. **26**: 1525-1530.

63. Crosatti, C., Polverino de Laureto, P., Bassi, R. et Cattivelli, L. 1999. The interaction between cold and light controls the expression of the cold-regulated barley gene cor14b and the accumulation of the corresponding protein. Plant Physiol. **119**: 671-680.

64. Curtis, P.S.W., X.Z. . 1998. A meta-analysis of elevated CO2 effects on woody plants mass, form, and physiology. Oecoligia **113**: 299-313.

65. Dahal, K., Gadapati, W., Savitch, L.V., Singh, J. et Hüner, N.P. 2012. Cold acclimation and BnCBF17-over-expression enhance photosynthetic performance and energy conversion efficiency during long-term growth of *Brassica napus* under elevated CO_2 conditions. Planta **236**: 1639-1652.

66. Dahal, K., Kane, K., Gadapati, W., Webb, E., Savitch, L.V., Singh, J., Sharma, P., Sarhan, F., Longstaffe, F.J., Grodzinski, B. et Hüner, N.P. 2012. The effects of phenotypic plasticity on photosynthetic performance in winter rye, winter wheat and *Brassica napus*. Physiol Plant **144**: 169-188.

67. Dahal, K., Kane, K., Sarhan, F., Grodzinski, B. et Hüner, N.P.A. 2012. Cold acclimation inhibits CO2-dependent stimulation of photosynthesis in spring wheat and spring rye. Botany **90**: 433-444.

68. Dalen, L.S. et Johnsen, ÿ. 2004. CO2 enrichment, nitrogen fertilization and development of freezing tolerance in *Norway spruce*. Trees-Structure and Function **18**: 10-18.

69. Danyluk, J., Perron, A., Houde, M., Limin, A., Fowler, B., Benhamou, N. et Sarhan, F. 1998. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell **10**: 623-638.

70. Danyluk, J., Perron, A., Houde, M., Limin, A., Fowler, B., Benhamou, N. et Sarhan, F. 1998. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. Plant Cell **10**: 623-638.

71. Davey, P.A., Hunt, S., Hymus, G.J., DeLucia, E.H., Drake, B.G., Karnosky, D.F. et Long, S.P. 2004. Respiratory oxygen uptake is not decreased by an instantaneous elevation of [CO2], but is increased with long-term growth in the field at elevated [CO2]. Plant Physiol. **134**: 520.

72. Davidson, R.M., Reeves, P.A., Manosalva, P.M. et Leach, J.E. 2009. Germins: A diverse protein family important for crop improvement. Plant Sci. **177**: 499-510.

73. De Lucia, E., Nabity, P., Zavala, J. et Berenbaum, M. 2012. Climate Change: Resetting Plant-Insect Interactions. Plant Physiol.).

74. DeFalco, T., Bender, K. et Snedden, W. 2010. Breaking the code: Ca2+ sensors in plant signalling. Biochem. J **425**: 27-40.

75. Delucia, E.H., Sasek, T.W. et Strain, B.R. 1985. Photosynthetic inhibition after long-term exposure to elevated levels of atmospheric carbon dioxide. Photosynthesis Res. **7**: 175-184.

76. Demmig-Adams, B. et Adams III, W. 1992. Photoprotection and other responses of plants to high light stress. Annu. Rev. Plant Biol. **43**: 599-626.

77. Dhont, C.C., Nadeau, Y., Bélanger, P. et Chalifour, G. 2003. Alfalfa root nitrogen reserves and regrowth potential in response to fall harvests. Crop Sci. **43**: 181-194.

78. Di Toppi, L.S., Marabottini, R., Badiani, M. et Raschi, A. 2002. Antioxidant status in herbaceous plants growing under elevated CO_2 in mini-FACE rings. J. Plant Physiol. **159**: 1005-1013

79. Doherty, C.J., Van Buskirk, H.A., Myers, S.J. et Thomashow, M.F. 2009. Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. Plant Cell **21**: 972-984.

80. Drake, B.G., Gonzàlez-Meler, M.A. et Long, S.P. 1997. More efficient plants: a consequence of rising atmospheric CO_2? Annu. Rev. Plant Biol. **48**: 609-639.

81. Eckey, C., Korell, M., Leib, K., Biedenkopf, D., Jansen, C., Langen, G. et Kogel, K.H. 2004. Identification of powdery mildew-induced barley genes by

cDNA-AFLP: functional assessment of an early expressed MAP kinase. Plant Mol. Biol. **55**: 1-15.

82. Ensminger, I., Busch, F. et Hüner, N. 2006. Photostasis and cold acclimation: sensing low temperature through photosynthesis. Physiol. Plant. **126**: 28-44.

83. Epron, D., Godard, D., Cornic, G. et Genty, B. 2006. Limitation of net CO2 assimilation rate by internal resistances to CO2 transfer in the leaves of two tree species (*Fagus sylvatica* L. and *Castanea sativa Mill.*). Plant, Cell Environ. **18**: 43-51.

84. Eulgem, T., Rushton, P.J., Robatzek, S. et Somssich, I.E. 2000. The WRKY superfamily of plant transcription factors. Trends Plant Sci. **5**: 199-205.

85. Farquhar, G., Caemmerer, S. et Berry, J. 1980. A biochemical model of photosynthetic CO 2 assimilation in leaves of C 3 species. Planta **149**: 78-90.

86. Farquhar, G.D. 1997. Carbon dioxide and vegetation. Science **278**: 1411.

87. Farrar, J., Pollock, C. et Gallagher, J. 2000. Sucrose and the integration of metabolism in vascular plants. Plant Sci. **154**: 1-11.

88. Ferrer, J.L., Jez, J.M., Bowman, M.E., Dixon, R.A. et Noel, J.P. 1999. Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat Struct Biol **6**: 775-784.

89. Flexas, J., Ribas-Carbo, M., Hanson, D.T., Bota, J., Otto, B., Cifre, J., McDowell, N., Medrano, H. et Kaldenhoff, R. 2006. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 in vivo. Plant J. **48**: 427-439.

90. Fowler, D. et Tyler, L. 1981. Selection for winterhardiness in wheat. III. Screening methods. Crop Sci. **21**: 896.

91. Foyer, C., Furbank, R., Harbinson, J. et Horton, P. 1990. The mechanisms contributing to photosynthetic control of electron transport by carbon assimilation in leaves. Photosynthesis Res. **25**: 83-100.

92. Foyer, C.H., Lelandais, M. et Kunert, K.J. 1994. Photooxidative stress in plants. Physiol. Plant. **92**: 696-717.

93. Franklin, K.A. 2009. Light and temperature signal crosstalk in plant development. Curr. Opin. Plant Biol. **12**: 63-68.

94. Fukayama, H., Fukuda, T., Masumoto, C., Taniguchi, Y., Sakai, H., Cheng, W., Hasegawa, T. et Miyao, M. 2009. Rice plant response to long term CO_2 enrichment: Gene expression profiling. Plant Sci. **177**: 203-210.

95. Gillespie, K.M., Rogers, A. et Ainsworth, E.A. 2011. Growth at elevated ozone or elevated carbon dioxide concentration alters antioxidant capacity and response to acute oxidative stress in soybean (*Glycine max*). J. Exp. Bot. **62**: 2667-2678.

96. Gilmour, S.J., Fowler, S.G. et Thomashow, M.F. 2004. Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol. Biol. **54**: 767-781.

97. Gilmour, S.J., Sebolt, A.M., Salazar, M.P., Everard, J.D. et Thomashow, M.F. 2000. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. **124**: 1854-1865.

98. Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M. et Thomashow, M.F. 1998. Low temperature regulation of theArabidopsis CBF family of AP2 transcriptional activators as an early step in cold induced COR gene expression. Plant J. **16**: 433-442.

99. Gjetting, T., Hagedorn, P.H., Schweizer, P., Thordal-Christensen, H., Carver, T.L. et Lyngkjaer, M.F. 2007. Single-cell transcript profiling of barley attacked by the powdery mildew fungus. Mol Plant Microbe Interact **20**: 235-246.

100. Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. et Ryals, J. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell **8**: 629-643.

101. Gorsuch, P.A., Pandey, S. et Atkin, O.K. 2010. Temporal heterogeneity of cold acclimation phenotypes in Arabidopsis leaves. Plant Cell Environ **33**: 244-258.

102. Gorsuch, P.A., Pandey, S. et Atkin, O.K. 2010. Thermal de-acclimation: how permanent are leaf phenotypes when cold-acclimated plants experience warming? Plant Cell Environ **33**: 1124-1137.

103. Gray, G.R., Chauvin, L.P., Sarhan, F. et Hüner, N.P.A. 1997. Cold acclimation and freezing tolerance (A complex interaction of light and temperature). Plant Physiol. **114**: 467.

104. Gray, G.R., Savitch, L.V., Ivanov, A.G. et Hüner, N. 1996. Photosystem II Excitation Pressure and Development of Resistance to Photoinhibition (II. Adjustment of Photosynthetic Capacity in Winter Wheat and Winter Rye). Plant Physiol **110**: 61-71.

105. Gray, J.E., Holroyd, G.H., van der Lee, F.M., Bahrami, A.R., Sijmons, P.C., Woodward, F.I., Schuch, W. et Hetherington, A.M. 2000. The HIC signalling pathway links CO2 perception to stomatal development. Nature **408**: 713-716.

106. Griffith, M. et Yaish, M.W. 2004. Antifreeze proteins in overwintering plants: a tale of two activities. Trends Plant Sci. **9**: 399-405.

107. Gupta, P., Duplessis, S., White, H., Karnosky, D., Martin, F. et Podila, G. 2005. Gene expression patterns of trembling aspen trees following long term exposure to interacting elevated CO2 and tropospheric O3. New Phytol. **167**: 129-142.

108. Guy, C., Niemi, K.J. et Brambl, R. 1985. Altered gene expression during cold acclimation of spinach. Proc. Natl. Acad. Sci. USA **82**: 3673.

109. Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**: 187-223.

110. Guy, C.L., Huber, J.L.A. et Huber, S.C. 1992. Sucrose phosphate synthase and sucrose accumulation at low temperature. Plant Physiol. **100**: 502-508.

111. Hannah, M.A., Heyer, A.G. et Hincha, D.K. 2005. A global survey of gene regulation during cold acclimation in Arabidopsis thaliana. PLoS Genet. **1**: e26.

112. Hanstein, S.M. et Felle, H.H. 2002. CO2-triggered chloride release from guard cells in intact fava bean leaves. Kinetics of the onset of stomatal closure. Plant Physiol. **130**: 940.

113. Harley, P.C. et Sharkey, T.D. 1991. An improved model of C3 photosynthesis at high CO_2: reversed O_2 sensitivity explained by lack of glycerate reentry into the chloroplast. Photosynthesis Res. **27**: 169-178.

114. Hashimoto, M., Negi, J., Young, J., Israelsson, M., Schroeder, J.I. et Iba, K. 2006. Arabidopsis HT1 kinase controls stomatal movements in response to CO2. Nat Cell Biol **8**: 391-397.

115. Hedrich, R. et Marten, I. 1993. Malate-induced feedback regulation of plasma membrane anion channels could provide a CO2 sensor to guard cells. EMBO Journal **12**: 897-901.

116. Heinze, B. 2007. A database of PCR primers for the chloroplast genomes of higher plants. Plant Methods **3**: 4.

117. Hetherington, A.M. et Woodward, F.I. 2003. The role of stomata in sensing and driving environmental change. Nature **424**: 901-908.

118. Higgins, S.I. et Scheiter, S. 2012. Atmospheric CO2 forces abrupt vegetation shifts locally, but not globally. Nature **488**: 209-212.

119. Hiilovaara-Teijo, M., Hannukkala, A., Griffith, M., Yu, X.M. et Pihakaski-Maunsbach, K. 1999. Snow-mold-induced apoplastic proteins in winter rye leaves lack antifreeze activity. Plant Physiol **121**: 665-674.

120. Himmelbach, A. 1998. Signalling of abscisic acid to regulate plant growth. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences **353**: 1439.

121. Hôgy, P., Wieser, H., Köhler, P., Schwadorf, K., Breuer, J., Franzaring, J., Muntifering, R. et Fangmeier, A. 2009. Effects of elevated CO2 on grain yield and quality of wheat: results from a 3 year free air CO2 enrichment experiment. Plant Biol. **11**: 60-69.

122. Houde, M., Dhindsa, R.S. et Sarhan, F. 1992. A molecular marker to select for freezing tolerance in Gramineae. Mol Gen Genet **234**: 43-48.

123. Houghton, J.T., Ding, Y., Griggs, D.J., Noguer, M., van der LINDEN, P.J., Dai, X., Maskell, K. et Johnson, C. 2001. *Climate change 2001: the scientific basis*, 881, Cambridge University Press Cambridge.

124. Huner, N. 1985. Morphological, anatomical, and molecular consequences of growth and development at low temperature in Secale cereale L. cv. Puma. Am. J. Bot.): 1290-1306.

125. Huner, N., √ñquist, G. et Sarhan, F. 1998. Energy balance and acclimation to light and cold. Trends Plant Sci. **3**: 224-230.

126. Hüner, N., Öquist, G., Hurry, V., Kröl, M., Falk, S. et Griffith, M. 1993. Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. Photosynthesis Res. **37**: 19-39.

127. Huner, N., Palta, J., Li, P. et Carter, J. 1981. Anatomical changes in leaves of Puma rye in response to growth at cold-hardening temperatures. Botanical Gazette): 55-62.

128. Huner, N.P.A., √ñquist, G., Hurry, V.M., Krol, M., Falk, S. et Griffith, M. 1993. Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. Photosynthesis Res. **37**: 19-39.

129. Huner, N.P.A., Elfman, B., Krol, M. et McIntosh, A. 1984. Growth and development at cold-hardening temperatures. Chloroplast ultrastructure, pigment content, and composition. Canadian journal of botany **62**: 53-60.

130. Huner, N.P.A., Palta, J.P., Li, P.H. et Carter, J.V. 1981. Comparison of the structure and function of ribulosebisphosphate carboxylase-oxygenase from a cold-hardy and nonhardy potato species. Can. J. Biochem. **59**: 280-289.

131. Hurry, V., Tobiaeson, M., Kr√∂mer, S., Gardestr√∂m, P. et √ñquist, G. 2006. Mitochondria contribute to increased photosynthetic capacity of leaves of winter rye (*Secale cereale* L.) following cold-hardening. Plant, Cell Environ. **18**: 69-76.

132. Hurry, V.M. et Hüner, N. 1991. Low growth temperature effects a differential inhibition of photosynthesis in spring and winter wheat. Plant Physiol. **96**: 491.

133. Hurry, V.M. et Huner, N.P.A. 1992. Effect of cold hardening on sensitivity of winter and spring wheat leaves to short-term photoinhibition and recovery of photosynthesis. Plant Physiol. **100**: 1283-1290.

134. Hurry, V.M. et Hüner, N.P.A. 1992. Effect of cold hardening on sensitivity of winter and spring wheat leaves to short-term photoinhibition and recovery of photosynthesis. Plant Physiol **100**: 1283-1290.

135. Hurry, V.M., Krol, M., Öquist, G. et Hüner, N.P.A. 1992. Effect of long-term photoinhibition on growth and photosynthesis of cold-hardened spring and winter wheat. Planta **188**: 369-375.

136. Hurry, V.M., Malmberg, G., Gardestrom, P. et Öquist, G. 1994. Effects of a short-term shift to low temperature and of long-term cold hardening on photosynthesis and ribulose-1,5-bisphosphate carboxylase/oxygenase and

sucrose phosphate synthase activity in leaves of winter rye (*Secale cereale* L.). Plant Physiol **106**: 983-990.

137. Hurry, V.M., Malmberg, G., Gardestrom, P. et Ōquist, G. 1994. Effects of a Short-Term Shift to Low Temperature and of Long-Term Cold Hardening on Photosynthesis and Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase and Sucrose Phosphate Synthase Activity in Leaves of Winter Rye (*Secale cereale L.*). Plant Physiol **106**: 983-990.

138. Hurry, V.M., Strand, A., Tobiaeson, M., Gardestrom, P. et Ōquist, G. 1995. Cold hardening of spring and winter wheat and rape results in differential effects on growth, carbon metabolism, and carbohydrate content. Plant Physiol **109**: 697-706.

139. Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K. et Yamaguchi-Shinozaki, K. 2006. Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant and Cell Physiology **47**: 141.

140. Jach, M. et Ceulemans, R. 2000. Short-versus long-term effects of elevated CO 2 on night-time respiration of needles of Scots pine (*Pinus sylvestris L.*). Photosynthetica **38**: 57-67.

141. Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T. et Thomashow, M.F. 2001. Components of the Arabidopsis C-Repeat/Dehydration-Responsive Element Binding Factor Cold-Response Pathway Are Conserved inBrassica napus and Other Plant Species. Plant Physiol. **127**: 910.

142. Jahnke, S. 2001. Atmospheric CO2 concentration does not directly affect leaf respiration in bean or poplar. Plant, Cell Environ. **24**: 1139-1151.

143. Jahnke, S. et Krewitt, M. 2002. Atmospheric CO2 concentration may directly affect leaf respiration measurement in tobacco, but not respiration itself*. Plant, Cell Environ. **25**: 641-651.

144. Jiang, C.J., Shimono, M., Maeda, S., Inoue, H., Mori, M., Hasegawa, M., Sugano, S. et Takatsuji, H. 2009. Suppression of the rice fatty-acid desaturase gene OsSSI2 enhances resistance to blast and leaf blight diseases in rice. Mol Plant Microbe Interact **22**: 820-829.

145. Jiang, C.J., Shimono, M., Maeda, S., Inoue, H., Mori, M., Hasegawa, M., Sugano, S. et Takatsuji, H. 2009. Suppression of the rice fatty-acid desaturase

gene OsSSI2 enhances resistance to blast and leaf blight diseases in rice. Mol. Plant-Microbe Interact. **22**: 820-829.

146. Kaiser, W.M. et Huber, S.C. 2001. Post translational regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. J. Exp. Bot. **52**: 1981.

147. Kane, N.A., Agharbaoui, Z., Diallo, A.O., Adam, H., Tominaga, Y., Ouellet, F. et Sarhan, F. 2007. TaVRT2 represses transcription of the wheat vernalization gene TaVRN1. Plant J. **51**: 670-680.

148. Kane, N.A., Danyluk, J., Tardif, G., Ouellet, F., Laliberte, J.F., Limin, A.E., Fowler, D.B. et Sarhan, F. 2005. TaVRT-2, a member of the StMADS-11 clade of flowering repressors, is regulated by vernalization and photoperiod in wheat. Plant Physiol. **138**: 2354.

149. Kaplan, F., Kopka, J., Sung, D.Y., Zhao, W., Popp, M., Porat, R. et Guy, C.L. 2007. Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold regulated gene expression with modifications in metabolite content. Plant J. **50**: 967-981.

150. Karlsson, M. et Stenlid, J. 2008. Comparative evolutionary histories of the fungal chitinase gene family reveal non-random size expansions and contractions due to adaptive natural selection. Evolutionary Bioinformatics Online **4**: 47-60.

151. Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. et Shinozaki, K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat. Biotechnol. **17**: 287-291.

152. Kim, T.H., Böhmer, M., Hu, H., Nishimura, N. et Schroeder, J.I. 2010. Guard cell signal transduction network: advances in understanding abscisic acid, CO2, and Ca2+ signaling. Annu. Rev. Plant Biol. **61**: 561.

153. Kimball, B., Kobayashi, K. et Bindi, M. 2002. Responses of agricultural crops to free-air CO2 enrichment. Advances in Agronomy **77**: 293-368.

154. Kimball, B., Morris, C., Pinter Jr, P., Wall, G., Hunsaker, D., Adamsen, F., LaMorte, R., Leavitt, S., Thompson, T. et Matthias, A. 2001. Elevated CO2, drought and soil nitrogen effects on wheat grain quality. New Phytol. **150**: 295-303.

155. Knight, C.A., DeVries, A.L. et Oolman, L.D. 1984. Fish antifreeze protein and the freezing and recrystallization of ice. Nature **308**: 295-296.

156. Kobayashi, F., Takumi, S., Nakata, M., Ohno, R., Nakamura, T. et Nakamura, C. 2004. Comparative study of the expression profiles of the Cor/Lea gene family in two wheat cultivars with contrasting levels of freezing tolerance. Physiol. Plant. **120**: 585-594.

157. Koch, K. 1996. Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Biol. **47**: 509-540.

158. Kočová, M., Holá, D., Wilhelmová, N. et Rothová, O. 2009. The influence of low-temperature on the photochemical activity of chloroplasts and activity of antioxidant enzymes in maize leaves. Biol. Plant. **53**: 475-483.

159. Krause, G.H. 1988. Photoinhibition of photosynthesis. An evaluation of damaging and protective mechanisms. Physiol. Plant. **74**: 566-574.

160. Kröl, M., Griffith, M. et Hüner, N. 1984. An appropriate physiological control for environmental temperature studies: comparative growth kinetics of winter rye. Canadian journal of botany **62**: 1062-1068.

161. Kumar, A., Li, C. et Portis, A.R., Jr. 2009. Arabidopsis thaliana expressing a thermostable chimeric Rubisco activase exhibits enhanced growth and higher rates of photosynthesis at moderately high temperatures. Photosynth Res **100**: 143-153.

162. Kuwabara, C. et Imai, R. 2009. Molecular basis of disease resistance acquired through cold acclimation in overwintering plants. Journal of plant biology **52**: 19-26.

163. Lal, M., Singh, K., Rathore, L., Srinivasan, G. et Saseendran, S. 1998. Vulnerability of rice and wheat yields in NW India to future changes in climate. Agricultural and Forest Meteorology **89**: 101-114.

164. Laohavisit, A. et Davies, J.M. 2009. Multifunctional annexins. Plant Sci. **177**: 532-539.

165. Lapointe, L. et Huner, N. 2006. Photoinhibition of isolated mesophyll cells from cold,Äêhardened and non,Äêhardened winter rye. Plant, Cell Environ. **16**: 249-258.

166. Leakey, A.D., Ainsworth, E.A., Bernacchi, C.J., Rogers, A., Long, S.P. et Ort, D.R. 2009. Elevated CO2 effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. J Exp Bot **60**: 2859-2876.

167. Leavitt, S.W., Paul, E.A., Galadima, A., Nakayama, F.S., Danzer, S.R., Johnson, H. et Kimball, B.A. 1995. Carbon isotopes and carbon turnover in cotton and wheat FACE experiments. Plant Soil **187**: 147-155.

168. Lee, B., Henderson, D.A. et Zhu, J.K. 2005. The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. Plant Cell **17**: 3155-3175.

169. Lee, C.M. et Thomashow, M.F. 2012. Photoperiodic regulation of the C-repeat binding factor (CBF) cold acclimation pathway and freezing tolerance in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **109**: 15054-15059.

170. Lee, M., Choi, Y., Burla, B., Kim, Y.Y., Jeon, B., Maeshima, M., Yoo, J.Y., Martinoia, E. et Lee, Y. 2008. The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to CO2. Nat. Cell Biol. **10**: 1217-1223.

171. Lee, T.D., Tjoelker, M.G., Ellsworth, D.S. et Reich, P.B. 2001. Leaf gas exchange responses of 13 prairie grassland species to elevated CO_2 and increased nitrogen supply. New Phytol. **150**: 405-418

172. Leonardos, E.D., Savitch, L.V., Huner, N.P., Oquist, G. et Grodzinski, B. 2003. Daily photosynthetic and C-export patterns in winter wheat leaves during cold stress and acclimation. Physiol Plant **117**: 521-531.

173. Li, P., Ainsworth, E.A., Leakey, A.D., Ulanov, A., Lozovaya, V., Ort, D.R. et Bohnert, H.J. 2008. Arabidopsis transcript and metabolite profiles: ecotype-specific responses to open-air elevated (CO_2). Plant, Cell Environ. **31**: 1673-1687.

174. Li, Z., Wakao, S., Fischer, B.B. et Niyogi, K.K. 2009. Sensing and responding to excess light. Annu Rev Plant Biol **60**: 239-260.

175. Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. et Shinozaki, K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10**: 1391-1406.

176. Livak, K.J. et Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**: 402-408.

177. Lobell, D.B., Ortiz-Monasterio, J.I., Asner, G.P., Matson, P.A., Naylor, R.L. et Falcon, W.P. 2005. Analysis of wheat yield and climatic trends in Mexico. Field Crops Res. **94**: 250-256.

178. Long, S.P., Ainsworth, E.A., Rogers, A. et Ort, D.R. 2004. Rising atmospheric carbon dioxide: plants FACE the future. Annu Rev Plant Biol **55**: 591-628.

179. Margolis, H.A. et Vézina, L.P. 1990. Atmospheric CO2 enrichment and the development of frost hardiness in containerized black spruce seedlings. Can. J. For. Res **20**: 1392-1398.

180. Mariscal, V., Moulin, P., Orsel, M., Miller, A.J., Fernández, E. et Galván, A. 2006. Differential regulation of the Chlamydomonas Nar1 gene family by carbon and nitrogen. Protist **157**: 421-433.

181. Marocco, A., Wissenbach, M., Becker, D., Paz-Ares, J., Saedler, H., Salamini, F. et Rohde, W. 1989. Multiple genes are transcribed in *Hordeum vulgare* and *Zea mays* that carry the DNA binding domain of the myb oncoproteins. Mol Gen Genet **216**: 183-187.

182. Martin, M., Gavazov, K., Koerner, C., Haettenschwiler, S. et Rixen, C. 2010. Reduced early growing season freezing resistance in alpine treeline plants under elevated atmospheric CO_2. Global Change Biol. **16**: 1057-1070.

183. Maruyama, K., Takeda, M., Kidokoro, S., Yamada, K., Sakuma, Y., Urano, K., Fujita, M., Yoshiwara, K., Matsukura, S. et Morishita, Y. 2009. Metabolic pathways involved in cold acclimation identified by integrated analysis of metabolites and transcripts regulated by DREB1A and DREB2A. Plant Physiol. **150**: 1972.

184. Mauch, F., Mauch-Mani, B. et Boller, T. 1988. Antifungal hydrolases in pea tissue : II. Inhibition of fungal growth by combinations of chitinase and beta-1,3-Glucanase. Plant Physiol **88**: 936-942.

185. Maurel, C., Verdoucq, L., Luu, D.T. et Santoni, V. 2008. Plant aquaporins: membrane channels with multiple integrated functions. Annu Rev Plant Biol **59**: 595-624.

186. McAinsh, M.R., Clayton, H., Mansfield, T.A. et Hetherington, A.M. 1996. Changes in Stomatal Behavior and Guard Cell Cytosolic Free Calcium in Response to Oxidative Stress. Plant Physiol **111**: 1031-1042.

187. McDonald, A.E., Ivanov, A.G., Bode, R., Maxwell, D.P., Rodermel, S.R. et H√°ner, N. 2011. Flexibility in photosynthetic electron transport: The physiological role of plastoquinol terminal oxidase (PTOX). Biochimica et Biophysica Acta (BBA)-Bioenergetics **1807**: 954-967.

188. Michaels, S.D. et Amasino, R.M. 1999. Flowering locus C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11**: 949.

189. Monroy, A.F., Dryanova, A., Malette, B., Oren, D.H., Ridha Farajalla, M., Liu, W., Danyluk, J., Ubayasena, L.W.C., Kane, K. et Scoles, G.J. 2007. Regulatory gene candidates and gene expression analysis of cold acclimation in winter and spring wheat. Plant Mol. Biol. **64**: 409-423.

190. Monroy, A.F., Labbé, E. et Dhindsa, R.S. 1997. Low temperature perception in plants: effects of cold on protein phosphorylation in cell-free extracts. FEBS Lett. **410**: 206-209.

191. Monte, E., Al-Sady, B., Leivar, P. et Quail, P.H. 2007. Out of the dark: how the PIFs are unmasking a dual temporal mechanism of phytochrome signalling. J Exp Bot **58**: 3125-3133.

192. Moore, B., Cheng, S.H., Rice, J. et Seemann, J. 1998. Sucrose cycling, Rubisco expression, and prediction of photosynthetic acclimation to elevated atmospheric CO_2. Plant, Cell Environ. **21**: 905-915.

193. Moore, B., Cheng, S.H., Sims, D. et Seemann, J. 1999. The biochemical and molecular basis for photosynthetic acclimation to elevated atmospheric CO2. Plant, Cell Environ. **22**: 567-582.

194. Morgan, J.A., LeCain, D.R., Pendall, E., Blumenthal, D.M., Kimball, B.A., Carrillo, Y., Williams, D.G., Heisler-White, J., Dijkstra, F.A. et West, M. 2011. C4 grasses prosper as carbon dioxide eliminates desiccation in warmed semi-arid grassland. Nature **476**: 202-205.

195. Mortimer, J.C., Laohavisit, A., Macpherson, N., Webb, A., Brownlee, C., Battey, N.H. et Davies, J.M. 2008. Annexins: multifunctional components of growth and adaptation. J. Exp. Bot. **59**: 533-544.

196. Mott, K.A., Sibbernsen, E.D. et Shope, J.C. 2008. The role of the mesophyll in stomatal responses to light and CO2. Plant cell and Envrionment **31**: 1299-1306.

197. Muthukrïshnan, S., Liang, G.H., Trick, H.N. et Gill, B.S. 2001. Pathogenesis-related proteins and their genes in cereals. Plant Cell Tiss. Org. Cult. **64**: 93-114

198. Nakamura, T., Ohta, M., Sugiura, M. et Sugita, M. 2001. Chloroplast ribonucleoproteins function as a stabilizing factor of ribosome-free mRNAs in the stroma. J Biol Chem **276**: 147-152.

199. NDong, C., Anzellotti, D., Ibrahim, R.K., Huner, N.P. et Sarhan, F. 2003. Daphnetin methylation by a novel O-methyltransferase is associated with cold acclimation and photosystem II excitation pressure in rye. J. Biol. Chem. **278**: 6854-6861.

200. NDong, C., Danyluk, J., Wilson, K.E., Pocock, T., Hüner, N.P.A. et Sarhan, F. 2002. Cold-regulated cereal chloroplast late embryogenesis abundant-like proteins. Molecular characterization and functional analyses. Plant Physiol **129**: 1368-1381.

201. Nie, G., Hendrix, D.L., Webber, A.N., Kimball, B.A. et Long, S.P. 1995. Increased accumulation of carbohydrates and decreased photosynthetic gene transcript levels in wheat grown at an elevated CO2 concentration in the field. Plant Physiol. **108**: 975.

202. Norby, R.J., Hanson, P.J., O'Neill, E.G., Tschaplinski, T.J., Weltzin, J.F., Hansen, R.A., Cheng, W., Wullschleger, S.D., Gunderson, C.A. et Edwards, N.T. 2002. Net primary productivity of a CO2-enriched deciduous forest and the implications for carbon storage. Ecol. Appl. **12**: 1261-1266.

203. O'Leary, M.H. 1988. Carbon isotopes in photosynthesis. Bioscience): 328-336.

204. Obrist, D., Arnone, J. et K√∂rner, C. 2001. In situ Effects of Elevated Atmospheric CO2on Leaf Freezing Resistance and Carbohydrates in a Native Temperate Grassland. Ann. Bot. **87**: 839-844.

205. Ogo, Y., Itai, R.N., Nakanishi, H., Inoue, H., Kobayashi, T., Suzuki, M., Takahashi, M., Mori, S. et Nishizawa, N.K. 2006. Isolation and characterization of IRO2, a novel iron-regulated bHLH transcription factor in graminaceous plants. J Exp Bot **57**: 2867-2678.

206. Öquist, G. et Hüner, N.P.A. 2003. Photosynthesis of overwintering evergreen plants. Annu Rev Plant Biol **54**: 329-355.

207. Öquist, G., Hurry, V.M. et Hüner, N.P.A. 1993. Low-temperature effects on photosynthesis and correlation with freezing tolerance in spring and winter cultivars of wheat and rye. Plant Physiol **101**: 245-250.

208. Ort, D.R. 2001. When there is too much light. Plant Physiol. **125**: 29-32.

209. Ort, D.R. et Yocum, C.F. 1996. *Oxygenic photosynthesis: the light reactions*, 4, Springer Netherlands.

210. Orvar, B.L., Sangwan, V., Omann, F. et Dhindsa, R.S. 2000. Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. Plant J. **23**: 785-794.

211. Ouellet, F., Vazquez-Tello, A. et Sarhan, F. 1998. The wheat wcs120 promoter is cold-inducible in both monocotyledonous and dicotyledonous species. FEBS Lett. **423**: 324-328.

212. Palet, A., Ribas-Carbó, M., Argilés, J.M. et Azcón-Bieto, J. 1991. Short-term effects of carbon dioxide on carnation callus cell respiration. Plant Physiol. **96**: 467.

213. Peet, M.M., Huber, S.C. et Patterson, D.T. 1986. Acclimation to High CO2 in *Monoecious Cucumbers*: II. Carbon Exchange Rates, Enzyme Activities, and Starch and Nutrient Concentrations. Plant Physiol. **80**: 63.

214. Pennycooke, J.C., Jones, M.L. et Stushnoff, C. 2003. Down-regulating a-galactosidase enhances freezing tolerance in transgenic Petunia. Plant Physiol. **133**: 901-909.

215. Pihakaski-Maunsbach, K., Griffith, M., Antikainen, M. et Maunsbach, A. 1996. Immunogold localization of glucanase-like antifreeze protein in cold acclimated winter rye. Protoplasma **191**: 115-125.

216. Pocock, T.H., Hurry, V., Savitch, L.V. et Hüner, N. 2001. Susceptibility to low temperature photoinhibition and the acquisition of freezing tolerance in winter and spring wheat: The role of growth temperature and irradiance. Physiol. Plant. **113**: 499-506.

217. Polle, A., Eiblmeier, M., Sheppard, L. et Murray, M. 1997. Responses of antioxidative enzymes to elevated CO_2 in leaves of beech (*Fagus sylvatica* L.)

seedlings grown under a range of nutrient regimes. Plant, Cell Environ. **20**: 1317-1321.

218. Ponce-Valadez, M., Fellman, S.M., Giovannoni, J., Gan, S.S. et Watkins, C.B. 2009. Differential fruit gene expression in two strawberry cultivars in response to elevated CO_2 during storage revealed by a heterologous fruit microarray approach. Postharvest Biol. Technol. **51**: 131-140.

219. Portis, A.R. 2003. Rubisco activase-Rubisco's catalytic chaperone. Photosynthesis Res. **75**: 11-27.

220. Pötter, E. et Kloppstech, K. 1993. Effects of light stress on the expression of early light-inducible proteins in barley. Eur. J. Biochem. **214**: 779-786

221. Pritchard, S.G., Ju, Z., Santen, V.E., QiuD, J., Weaver, D.B., Prior, S.A. et Rogers, H.H. 2000. The influence of elevated CO_2 on the activities of antioxidative enzymes in two soybean genotypes. Aust. J. Plant Physiol. **27**: 1061-1068.

222. Qiao, Y., Zhang, H., Dong, B., Shi, C., Li, Y., Zhai, H. et Liu, M. 2010. Effects of elevated CO2 concentration on growth and water use efficiency of winter wheat under two soil water regimes. Agric. Water Manage. **97**: 1742-1748.

223. Quirino, B.F., Noh, Y.S., Himelblau, E. et Amasino, R.M. 2000. Molecular aspects of leaf senescence. Trends Plant Sci. **5**: 278-282.

224. Rao, M.V., Hale, B.A. et Ormrod, D.P. 1995. Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide (role of antioxidant enzymes). Plant Physiol **109**: 421-432.

225. Rapacz, M., Wolanin, B., Hura, K. et Tyrka, M. 2008. The effects of cold acclimation on photosynthetic apparatus and the expression of COR14b in four genotypes of barley (Hordeum vulgare) contrasting in their tolerance to freezing and high-light treatment in cold conditions. Ann. Bot. **101**: 689-699.

226. Raschke, K., Shabahang, M. et Wolf, R. 2003. The slow and the quick anion conductance in whole guard cells: their voltage-dependent alternation, and the modulation of their activities by abscisic acid and CO2. Planta **217**: 639-650.

227. Rathnam, C. et Edwards, G.E. 1976. Distribution of nitrate-assimilating enzymes between mesophyll protoplasts and bundle sheath cells in leaves of three groups of C4 plants. Plant Physiol. **57**: 881-885.

228. Reuveni, J. et Gale, J. 1985. The effect of high levels of carbon dioxide on dark respiration and growth of plants. Plant, Cell Environ. **8**: 623-628.

229. Ristic, Z. et Ashworth, E.N. 1994. Response of xylem ray parenchyma cells of red osier dogwood (*Cornus sericea* L.) to freezing stress (microscopic evidence of protoplasm contraction). Plant Physiol. **104**: 737-746.

230. Rochaix, J.D. 1995. Chlamydomonas reinhardtii as the photosynthetic yeast. Annu Rev Genet **29**: 209-230.

231. Roelfsema, M., Konrad, K.R., Marten, H., Psaras, G.K., Hartung, W. et Hedrich, R. 2006. Guard cells in albino leaf patches do not respond to photosynthetically active radiation, but are sensitive to blue light, CO2 and abscisic acid. Plant, Cell Environ. **29**: 1595-1605.

232. Rolland, F., Moore, B. et Sheen, J. 2002. Sugar sensing and signaling in plants. Plant Cell **14**: 185-205.

233. Romeis, T. 2001. Protein kinases in the plant defence response. Curr Opin Plant Biol **4**: 407-414.

234. Rook, F., Corke, F., Card, R., Munz, G., Smith, C. et Bevan, M.W. 2001. Impaired sucrose induction mutants reveal the modulation of sugar induced starch biosynthetic gene expression by abscisic acid signalling. Plant J. **26**: 421-433.

235. Rosso, D., Bode, R., Li, W., Kröl, M., Saccon, D., Wang, S., Schillaci, L.A., Rodermel, S.R., Maxwell, D.P. et Hüner, N.P.A. 2009. Photosynthetic redox imbalance governs leaf sectoring in the *Arabidopsis thaliana* variegation mutants immutans, spotty, var1, and var2. Plant Cell **21**: 3473-3492.

236. Rowland Bamford, A., Baker, J., Allen Jr, L. et Bowes, G. 1991. Acclimation of rice to changing atmospheric carbon dioxide concentration. Plant, Cell Environ. **14**: 577-583.

237. Sage, R.F., Sharkey, T.D. et Seemann, J.R. 1989. Acclimation of photosynthesis to elevated CO2 in five C3 species. Plant Physiol. **89**: 590-596.

238. Salvucci, M.E. et Crafts-Brandner, S.J. 2004. Inhibition of photosynthesis by heat stress: the activation state of Rubisco as a limiting factor in photosynthesis. Physiol Plant **120**: 179-186.

239. Sandve, S.R., Rudi, H., Asp, T. et Rognli, O.A. 2008. Tracking the evolution of a cold stress associated gene family in cold tolerant grasses. BMC Evol. Biol. **8**: 245.

240. Sangwan, V., Foulds, I., Singh, J. et Dhindsa, R.S. 2001. Cold activation of *Brassica napus* BN115 promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca2+ influx. Plant J. **27**: 1-12.

241. Sarhan, F., Ouellet, F. et Vazquez-Tello, A. 1997. The wheat wcs120 gene family. A useful model to understand the molecular genetics of freezing tolerance in cereals. Physiol. Plant. **101**: 439-445.

242. Sarhan, F. et Perras, M. 1987. Accumulation of a high molecular weight protein during cold hardening of wheat (*Triticum aestivum L.*). Plant Cell Physiol. **28**: 1173-1179.

243. Sassenrath, G. et Ort, D. 1990. The relationship between inhibition of photosynthesis at low temperature and the inhibition of photosynthesis after rewarming in chill-sensitive tomato. Plant Physiol. Biochem. **28**: 457-465.

244. Savitch, L., Leonardos, E., Krol, M., Jansson, S., Grodzinski, B., Hüner, N. et Öquist, G. 2002. Two different strategies for light utilization in photosynthesis in relation to growth and cold acclimation. Plant, Cell Environ. **25**: 761-771.

245. Savitch, L.V., Allard, G., Seki, M., Robert, L.S., Tinker, N.A., Hüner, N.P.A., Shinozaki, K. et Singh, J. 2005. The effect of overexpression of two Brassica CBF/DREB1-like transcription factors on photosynthetic capacity and freezing tolerance in *Brassica napus*. Plant Cell Physiol **46**: 1525-1539.

246. Savitch, L.V., Barker-Astrom, J., Ivanov, A.G., Hurry, V., Öquist, G., Hüner, N.P.A. et Gardeström, P. 2001. Cold acclimation of *Arabidopsis thaliana* results in incomplete recovery of photosynthetic capacity, associated with an increased reduction of the chloroplast stroma. Planta **214**: 295-303.

247. Savitch, L.V., Harney, T. et Huner, N.P.A. 2003. Sucrose metabolism in spring and winter wheat in response to high irradiance, cold stress and cold acclimation. Physiol. Plant. **108**: 270-278.

248. Saxe, H., Ellsworth, D.S. et Heat, J. 1998. Tree and forest functioning in an enriched CO2 atmosphere. New Phytol **139**: 395-436.

249. Schlumbaum, A., Mauch, F., Vögeli, U. et Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature **324**: 365-367.

250. Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M. et Waner, D. 2001. Guard cell signal transduction. Annu. Rev. Plant Biol. **52**: 627-658.

251. Schwanz, P. et Polle, A. 2001. Growth under elevated CO_2 ameliorates defenses against photo-oxidative stress in poplar (*Populus alba x tremula*). Environ Exp Bot **45**: 43-53.

252. Schwanz, P. et Polle, A. 2001. Growth under elevated CO_2 ameliorates defenses against photo-oxidative stress in poplar (*Populus alba x tremula*). Environ. Exp. Bot. **45**: 43-53

253. Shen, Q.H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ulker, B., Somssich, I.E. et Schulze-Lefert, P. 2007. Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science **315**: 1098-1103.

254. Simpson, G.G. 2004. The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. Curr. Opin. Plant Biol. **7**: 570-574.

255. Skinner, R.H., Morgan, J.A. et Hanson, J.D. 1999. Carbon and nitrogen reserve remobilization following defoliation: nitrogen and elevated CO2 effects. Crop Sci. **39**: 1749-1756.

256. Smeekens, S. 2000. Sugar-induced signal transduction in plants. Annu. Rev. Plant Biol. **51**: 49-81.

257. Soitamo, A.J., Piippo, M., Allahverdiyeva, Y., Battchikova, N. et Aro, E.M. 2008. Light has a specific role in modulating Arabidopsis gene expression at low temperature. BMC Plant Biol. **8**: 13.

258. Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M. et Miller, H.L. 2007. Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change, 2007. IPCC. Cambridge University Press, Cambridge, UK and New York, NY, USA).

259. Somersalo, S. et Krause, G. 1989. Photoinhibition at chilling temperature. Planta **177**: 409-416.

260. Springer, C.J., Orozco, R.A., Kelly, J.K. et Ward, J.K. 2008. Elevated CO2 influences the expression of floral initiation genes in Arabidopsis thaliana. New Phytol. **178**: 63-67.

261. Stafford, H.A. et Ibrahim, R.K. 1992. *Phenolic metabolism in plants*, 26, Plenum Press, New York (USA).

262. Steponkus, P.L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Annual Review of Plant Physiology **35**: 543-584.

263. Stitt, M. 1991. Rising CO2 levels and their potential significance for carbon flow in photosynthetic cells. Plant, Cell Environ. **14**: 741-762.

264. Stitt, M. et Hurry, V. 2002. A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. Curr Opin Plant Biol **5**: 199-206.

265. Stitt, M. et Krapp, A. 1999. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant, Cell Environ. **22**: 583-621.

266. Stitt, M. et Quick, W.P. 1989. Photosynthetic carbon partitioning: its regulation and possibilities for manipulation. Physiol. Plant. **77**: 633-641.

267. Strand, Å., Foyer, C.H., Gustafsson, P., Gardeström, P. et Hurry, V. 2003. Altering flux through the sucrose biosynthesis pathway in transgenic *Arabidopsis thaliana* modifies photosynthetic acclimation at low temperatures and the development of freezing tolerance. Plant, Cell Environ. **26**: 523-535.

268. Strand, A., Hurry, V., Gustafsson, P. et Gardeström, P. 2003. Development of *Arabidopsis thaliana* leaves at low temperatures releases the suppression of photosynthesis and photosynthetic gene expression despite the accumulation of soluble carbohydrates. Plant J. **12**: 605-614.

269. Strand, A., Hurry, V., Henkes, S., Hüner, N.P.A., Gustafsson, P., Gardeström, P. et Stitt, M. 1999. Acclimation of Arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol **119**: 1387-1398.

270. Sung, S. et Amasino, R.M. 2005. Remembering winter: toward a molecular understanding of vernalization. Annu. Rev. Plant Biol. **56**: 491-508.

271. Sutton, F., Chen, D.G., Ge, X. et Kenefick, D. 2009. Cbf genes of the Fr-A2 allele are differentially regulated between long-term cold acclimated crown tissue of freeze-resistant and - susceptible, winter wheat mutant lines. BMC Plant Biol. **9**: 34.

272. Suzuki, N. et Mittler, R. 2006. Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol. Plant. **126**: 45-51.

273. Svensson, J.T., Crosatti, C., Campoli, C., Bassi, R., Stanca, A.M., Close, T.J. et Cattivelli, L. 2006. Transcriptome analysis of cold acclimation in barley Albina and Xantha mutants. Plant Physiol. **141**: 257.

274. Taji, T., Ohsumi, C., Iuchi, S., Seki, M., Kasuga, M., Kobayashi, M., Yamaguchi Shinozaki, K. et Shinozaki, K. 2002. Important roles of drought and cold inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J. **29**: 417-426.

275. Talanova, V., Titov, A., Topchieva, L., Malysheva, I., Venzhik, Y.V. et Frolova, S. 2009. Expression of WRKY transcription factor and stress protein genes in wheat plants during cold hardening and ABA treatment. Russian Journal of Plant Physiology **56**: 702-708.

276. Talukdar, T., Gorecka, K.M., Carvalho-Niebel, F., Downie, J.A., Cullimore, J. et Pikula, S. 2009. Annexins-calcium-and membrane-binding proteins in the plant kingdom potential role in nodulation and mycorrhization in *Medicago truncatula*. Acta Biochim. Pol. **56**: 199-210.

277. Tao, Z., Liu, H., Qiu, D., Zhou, Y., Li, X., Xu, C. et Wang, S. 2009. A pair of allelic WRKY genes play opposite roles in rice-bacteria interactions. Plant Physiol. **151**: 936-948.

278. Taylor, G., Street, N.R., Tricker, P.J., Sjodin, A., Graham, L., Skogstrom, O., Calfapietra, C., Scarascia-Mugnozza, G. et Jansson, S. 2005. The transcriptome of Populus in elevated CO_2. New Phytol **167**: 143-154.

279. Tcherkez, G.G., Farquhar, G.D. et Andrews, T.J. 2006. Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. Proc. Natl. Acad. Sci. USA **103**: 7246-7251.

280. Terry, A.C., Quick, W.P. et Beerling, D.J. 2000. Long-term growth of ginkgo with CO2 enrichment increases leaf ice nucleation temperatures and limits recovery of the photosynthetic system from freezing. Plant Physiol. **124**: 183.

281. Theocharis, A., Clement, C. et Barka, E.A. 2012. Physiological and molecular changes in plants grown at low temperatures. Planta **235**: 1091-1105.

282. Thomashow, M.F. 1998. Role of cold-responsive genes in plant freezing tolerance. Plant Physiol **118**: 1-8.

283. Thomashow, M.F. 2001. So what's new in the field of plant cold acclimation? Lots! Plant Physiol. **125**: 89-93.

284. Thomma, B.P., Penninckx, I.A., Cammue, B.P. et Broekaert, W.F. 2001. The complexity of disease signaling in Arabidopsis. Curr Opin Immunol **13**: 63-68.

285. Tonkaz, T., Dogan, E. et Kocyigit, R. 2010. Impact of temperature change and elevated carbon dioxide on winter wheat (*Triticum Aestivum L.*) grown under semi-arid conditions. Bulgarian Journal of Agricultural Science **16**: 565-575.

286. Tremblay, K., Ouellet, F., Fournier, J., Danyluk, J. et Sarhan, F. 2005. Molecular characterization and origin of novel bipartite cold-regulated ice recrystallization inhibition proteins from cereals. Plant Cell Physiol **46**: 884-891.

287. Uehlein, N., Lovisolo, C., Siefritz, F. et Kaldenhoff, R. 2003. The tobacco aquaporin NtAQP1 is a membrane CO2 pore with physiological functions. Nature **425**: 734-737.

288. Vahisalu, T., Kollist, H., Wang, Y.F., Nishimura, N., Chan, W.Y., Valerio, G., Lamminm‰ki, A., BroschÈ, M., Moldau, H. et Desikan, R. 2008. SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature **452**: 487-491.

289. Van Buskirk, H.A. et Thomashow, M.F. 2006. Arabidopsis transcription factors regulating cold acclimation. Physiol. Plant. **126**: 72-80.

290. Van Loon, L.C., Rep, M. et Pieterse, C.M.J. 2006. Significance of inducible defense-related proteins in infected plants. Annu. Rev. Phytopathol. **44**: 135-162

291. Vaultier, M.N., Cantrel, C., Vergnolle, C., Justin, A.M., Demandre, C., Benhassaine-Kesri, G., Çiçek, D., Zachowski, A. et Ruelland, E. 2006. Desaturase mutants reveal that membrane rigidification acts as a cold perception mechanism upstream of the diacylglycerol kinase pathway in Arabidopsis cells. FEBS Lett. **580**: 4218-4223.

292. Vigers, A.J., Wiedemann, S., Roberts, W.K., Legrand, M., Selitrennikoff, C.P. et Fritig, B. 1992. Thaumatin-like pathogenesis-related proteins are antifungal. Plant Sci. **83**: 155-161.

293. von Caemmerer, S. et Quick, W.P. 2000. Rubisco: physiology in vivo. Photosynthesis: Physiology and metabolism **9**: 85-113.

294. Wang, X., Liu, W., Chen, X., Tang, C., Dong, Y., Ma, J., Huang, X., Wei, G., Han, Q. et Huang, L. 2010. Differential gene expression in incompatible interaction between wheat and stripe rust fungus revealed by cDNA-AFLP and comparison to compatible interaction. BMC Plant Biol. **10**: 9.

295. Wanner, L.A. et Junttila, O. 1999. Cold-induced freezing tolerance in Arabidopsis. Plant Physiol. **120**: 391.

296. Warren, C.R. et Dreyer, E. 2006. Temperature response of photosynthesis and internal conductance to CO2: results from two independent approaches. J Exp Bot **57**: 3057-3067.

297. Wayne, P., Reekie, E. et Bazzaz, F. 1998. Elevated CO 2 ameliorates birch response to high temperature and frost stress: implications for modeling climate-induced geographic range shifts. Oecologia **114**: 335-342.

298. Wiese, A., Gröner, F., Sonnewald, U., Deppner, H., Lerchl, J., Hebbeker, U., Flügge, U.I. et Weber, A. 1999. Spinach hexokinase I is located in the outer envelope membrane of plastids. FEBS Lett. **461**: 13-18.

299. Wilson, K.E., Ivanov, A.G., Öquist, G., Grodzinski, B., Sarhan, F. et Huner, N.P.A. 2006. Energy balance, organellar redox status, and acclimation to environmental stress. Botany **84**: 1355-1370.

300. Wissenbach, M., Uberlacker, B., Vogt, F., Becker, D., Salamini, F. et Rohde, W. 1993. Myb genes from *Hordeum vulgare*: tissue-specific expression of chimeric Myb promoter/Gus genes in transgenic tobacco. Plant J. **4**: 411-422.

301. Woodward, F.I., Lake, J.A. et Quick, W.P. 2002. Stomatal development and CO_2: ecological consequences. New Phytol. **153**: 477-484.

302. Wu, G., Chen, F.J., Ge, F. et Xiao, N.-W. 2011. Impacts of elevated CO_2 on expression of plant defensive compounds in Bt-transgenic cotton in response to infestation by cotton bollworm. Agric. For. Entomol. **13**: 77-82.

303. Xiang, D.J., Hu, X.Y., Zhang, Y. et Yin, K.D. 2008. Over-expression of ICE1 gene in transgenic rice improves cold tolerance. Rice Science **15**: 173-178.

304. Yaish, M.W.F., Doxey, A.C., McConkey, B.J., Moffatt, B.A. et Griffith, M. 2006. Cold-active winter rye glucanases with ice-binding capacity. Plant Physiol. **141**: 1459-1472.

305. Yang, T., Chaudhuri, S., Yang, L., Chen, Y. et Poovaiah, B. 2004. Calcium/calmodulin up-regulates a cytoplasmic receptor-like kinase in plants. J. Biol. Chem. **279**: 42552-42559.

306. Yeh, S., Moffatt, B.A., Griffith, M., Xiong, F., Yang, D.S., Wiseman, S.B., Sarhan, F., Danyluk, J., Xue, Y.Q., Hew, C.L., Doherty-Kirby, A. et Lajoie, G. 2000. Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiol **124**: 1251-1264.

307. Young, J.J., Mehta, S., Israelsson, M., Godoski, J., Grill, E. et Schroeder, J.I. 2006. CO2 signaling in guard cells: calcium sensitivity response modulation, a Ca^{2+}-independent phase, and CO2 insensitivity of the gca2 mutant. Proc. Natl. Acad. Sci. USA **103**: 7506-7511.

308. Zavala, J.A., Casteel, C.L., Delucia, E.H. et Berenbaum, M.R. 2008. Anthropogenic increase in carbon dioxide compromises plant defense against invasive insects. Proceedings of National Academy of Sciences, USA **105**: 5129-5133.

309. Zhang, D.Y., Chen, G.Y., Chen, J., Yong, Z.H., Zhu, J.G. et Xu, D.Q. 2009. Photosynthetic acclimation to CO_2 enrichment related to ribulose-1,5-bisphosphate carboxylation limitation in wheat. Photosynthetica **47**: 152-154.

310. Zhou, J.M., Gold, N.D., Martin, V.J., Wollenweber, E. et Ibrahim, R.K. 2006. Sequential O-methylation of tricetin by a single gene product in wheat. Biochim Biophys Acta **1760**: 1115-1124.

311. Zimmermann, G., Bäumlein, H., Mock, H.P., Himmelbach, A. et Schweizer, P. 2006. The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. Plant Physiol. **142**: 181-192.